T LYMPHOCYTE RESPONSES TO MURINE CYTOMEGALOVIRUS INFECTION

by

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STATEMENT

All experiments described in this thesis represent my own work and were done by me.

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ABSTRACT

The work described in this thesis was undertaken to investigate one aspect of the immunobiology of murine cytomegalovirus (MCMV); the T cell response.

The starting point was the adaption of a murine model system described for Herpes simplex virus (Pfizenmaier et al. 1977) to the murine herpesvirus, MCMV. This consisted of hind footpad inoculation of virus into mice, followed by removal of draining popliteal lymph nodes, culture of the cell suspensions and assay for the presence of MCMV-specific effector cells. A reductionist approach was utilised for each step of the protocol to delineate some of the parameters that may affect the generation or detection of MCMV-specific cytotoxicity. The opportunity was also taken to examine another functional characteristic of T cells, that of lymphokine release.

In Chapter 2 initial observations demonstrated that after hind footpad inoculation of MCMV a virus-induced increase in viable cell numbers occurred within the draining popliteal lymph nodes, but the extent of the increase after day 2 post-inoculation was inversely related to virus dose. Harvest of lymph node cells from MCMV-infected mice and direct application onto target cells produced no detectable virus-specific cytotoxicity at 2, 4, 6, 8, 10, 12, 17 or 19 days post footpad inoculation, although lysis of uninfected syngeneic targets was obtained 4-8 days post-infection. Optimal cytotoxic activity against MCMV-infected target cells required harvest of draining popliteal lymph node cells 6-8 days

post footpad inoculation of virus followed by culture for 4 days. Injections of MCMV from $10^{2.6}$ up to $10^{6.1}$ plaque forming units into the footpad generated equivalent MCMV-specific cytotoxicity after 4 days of culture.

The MCMV-specific cytotoxicity was mediated by Thy1.2$^+$, Lyt2$^+$, Class I H-2 antigen restricted effector cells, properties characteristic of cytotoxic T cells. By manipulation of the *in vitro* conditions it was established that for optimal generation of cytotoxic T cells, cellular proliferation was necessary, that cellular proliferation was dependent upon Thy1.2$^+$, Lyt2$^+$ cell populations and that supernatants from Concanavalin-A activated spleen cells (CSS) enhanced the cytotoxicity levels obtained.

In Chapter 4 the MCMV-immune lymph node cells generated by the *in vivo-in vitro* protocol were examined for both cytotoxic activity against MCMV-infected mouse embryo fibroblasts (MEF) and the capacity to release a particular lymphokine, Interleukin-3, upon stimulation with the same MCMV-infected MEF. Cytotoxicity and lymphokine release were Class I, H-2 restricted, viral specific and dependent upon Thy1.2$^+$, Lyt2$^+$ cells. MCMV-immune T cells produced detectable levels of Interleukin-3 by one hour after mixing with syngeneic MCMV-infected MEF. The level of Interleukin-3 reached a plateau by 14 hours after stimulation. The stimulator ability of syngeneic MCMV-infected MEF was acquired at one hour after viral adsorption with a maximum ability attained by 8 hours.

In Chapter 5 MCMV-immune and alloreactive T cells were utilised to examine the conditions required to produce optimal target and stimulator cells. The performance of virus-specific targets was dependent upon cell type, cell culture conditions,

type of virus preparation and viral adsorption protocols. MEF cultured in medium supplemented with CSS and adsorbed with salivary gland preparations of MCMV were used either as target or stimulator cells and showed more susceptibility to lysis and more stimulator activity than MEF not treated with CSS.

CONTENTS

LIST OF ABBREVIATIONS

antibiotics	Streptomycin 200ug/ml, Penicillin G 100U/ml and Neomycin sulphate 125ug/ml
assay medium	EMEM + 5% FCS + antibiotics
B	Bursa dependent
C'	complement
^{51}Cr	$Na_2[^{51}Cr]O_4$
CSF	colony-stimulating factors
CSS	Concanavalin-A activated spleen cell supernatant
DMEM	Dulbecco's Modified Eagle's Medium supplemented with 5% FCS + antibiotics
EMEM	Eagle's Minimal Essential Medium
E:T	effector:target ratio
FCS	foetal calf serum
f.p.	footpad
HBSS	Hank's balanced salt solution
HI-FCS	heat-inactivated FCS
HSV	herpes simplex virus
Ig	Immunoglobulin
IL-1	Interleukin-1
IL-2	Interleukin-2
IL-3	Interleukin-3
i.p.	intraperitoneal
LAF	lymphocyte activating factor
LN	lymph nodes
MCMV	murine cytomegalovirus
2ME	2-mercaptoethanol

MEF	mouse embryo fibroblasts
MHC	major histocompatibility complex
MOI	multiplicity of infection
M.W.	molecular weight
P815	P-815-X2
p.f.u.	plaque forming units
S.D.	standard deviation
special gas	10% CO_2, 7% O_2 and 83% N_2
T	thymus dependent
Tc	cytotoxic T
TSF	thymocyte stimulating factor
UV	ultraviolet
VSV	vesicular stomatitis virus

CHAPTER 1

GENERAL INTRODUCTION

A. LYMPHOCYTES AND ADAPTIVE IMMUNITY

Introduction to the Immune System

The immune response is a mechanism for the removal of foreign material from a host. The vertebrate immune system is not only capable of a differential reaction (i.e., distinguishing between "self" and foreign material) leading to removal of an immense variety of foreign antigenic molecules but is also adaptive, in that subsequent encounters with the same foreign material will elicit a more rapid and vigorous response. Consequently, the immune system can be defined in terms of the reactive cells and proteins that specifically bind to foreign antigens, the induction requirements for these reactive cells and proteins,and the effector mechanisms mediated by these components that result in the elimination of foreign material. Various complex foreign materials, including transplanted tissue (Medawar 1944, 1945), tumours (Mitchison 1953), bacteria (Mackaness 1962, 1969) and viruses (Blanden 1974) as well as purified antigenic molecules (McDevitt & Chinitz 1969) have been utilized to study the mechanisms by which the adaptive immune system operates.

For experimentation I have chosen murine cytomegalovirus as an example of a particular foreign agent that can evade immunological elimination and cause persistent infection, but in the introduction, to illustrate specific aspects of the immune system, I will draw upon data obtained from other models. Furthermore, I will not discuss those host factors that may prevent interaction between virus and the components of the adaptive immune system (Mims 1979), that is, I wish to focus on those interactions that are subsequent to virus entry and replication in the host.

Early investigations of the adaptive immune system emphasised the specific reactive serum factors capable of effecting diphtheria and tetanus toxin neutralisation, lysis of certain micro-organisms in the presence of heat-labile serum factors, opsonisation of bacteria to augment phagocytosis and virus neutralisation (Root 1979). That cells were also a component of the adaptive immune response was evidenced by the adoptive transfer of tuberculin reactivity using viable lymphoid cells from immunised donors. In contrast, adoptive serum transfers did not initiate tuberculin reactivity (Bloom & Chase 1967). The general importance of cell-mediated effects was subsequently established with the different model systems; resistance to lymphosarcoma (Mitchison 1953, 1954), skin graft rejection (Billingham et al. 1954), graft versus host disease (Billingham & Brent 1959) and recovery from bacterial (Mackaness 1969) or viral infections (Blanden 1970, 1971a, 1971b).

Small lymphocytes initiate immune responses (Gowans et al. 1962, Gowans & McGregor 1965) and reside in the lymphoid system, but variation in the ability of different lymphoid organs to provide adoptive immune reactivity indicated that the lymphoid system was not uniform but was composed of heterologous cell populations (Billingham & Brent 1959). Furthermore different model systems varied with regard to which lymphoid organs behaved as sites containing reactive cells (Mitchison 1954, Billingham & Brent 1959).

Manipulations of lymphoid organs in chickens and mice provided proof of the existence of at least two categories of immunologically reactive cells, the Bursa dependent (B) cells and the thymus dependent (T) cells. Surgical or hormonal removal of a lymphoid outpouch found proximal to the chicken cloaca, the Bursa of Fabricius,

abolished antibody responses to foreign antigens (Graetzer et al. 1963) but not the ability to reject skin grafts (Szenberg & Warner 1962, Aspinall et al. 1963) whilst neonatal thymectomy resulted in animals unable to reject skin grafts (Miller 1962a, 1962b) but with intact antibody responses to foreign antigens (Aspinall et al. 1963, Graetzer et al. 1963). Concomitant investigations of immunological deficiency disease in humans, including agammaglobulinaemias, Wiskott-Aldrich syndrome and thymic aplasias, confirmed a similar T-B cell dichotomy in man (Good et al. 1962, Peterson et al. 1965, Rosen & Janeway 1966, Lischner et al. 1967). Although a Bursa of Fabricius has not been found in non-avian species, the combined data from other species revealed a lymphoid system composed of functionally different cells resposited within different organs and in different sites within the same organ (Cooper et al. 1965, Miller & Osaba 1967).

B lymphocytes

B lymphocytes are the precursors of antibody-forming plasma cells derived from stem cells in the yolk sac (Tyan & Herzenberg 1968), foetal liver and spleen (Nossal & Pike 1973) or bone marrow in adults (Mitchell & Miller 1968). The precursors mature into B cells and migrate to the secondary lymphoid organs such as spleen and peripheral lymph nodes (Kincade & Cooper 1971) to occupy the B-dependent areas.

B cells express a variety of cell surface markers including alloantigens such as Lyb4,5,6,7,8, heteroantigens, Fc and complement receptors (Katz 1977) but surface immunoglobulin (Ig) is the most widely used marker for identification. B cell subsets expressing

one or more of the known Ig classes namely IgM, IgD, IgG, IgA and IgE, have been identified (Nisonoff et al. 1975). IgM is the first Ig class expressed on B cells, subsequently followed by IgD (Kearney et al. 1977, Lala et al. 1979) and then other classes (Parkhouse & Cooper 1977). Usually the Ig receptor for antigen expressed on the B cell surface represents the specificity that will be synthesised following antigenic stimulation. However, B cells do have the ability to switch Ig class synthesised whilst maintaining the same antigen specificity (Nossal et al. 1964) and to undergo post-antigenic somatic rearrangement (Homuzi & Tonegawa 1976, Brack et al. 1978, Early et al. 1980) and mutation (Bothwell et al. 1981, Crews et al. 1981) of the genes coding for Ig antigen-binding sites.

T lymphocytes

Precursors of the thymus-dependent lymphoid cells also originate from haemopoietic tissues (Ford et al. 1956, Moore & Owen 1967). Stem cells migrate via the blood stream to the thymus (Gengozian et al. 1957, Metcalf & Wakonig-Vaartaja 1964, Harris et al. 1964) and differentiate into antigen-reactive T cells. Post-thymic T cells recirculate through blood, non-lymphoid tissues, lymph (Gowans 1959, Gowans & Knight 1964) and the thymus-dependent areas of the secondary lymphoid organs such as the spleen and lymph nodes (Parrot & DeSousa 1971).

As part of the differentiative process the cells acquire a variety of unique serologically detectable antigens on the cell membrane (McKenzie & Potter 1979). In the mouse, Thy1 was the first alloantigen used to distinguish T cells from B cells and is

now used as the standard cell surface marker for murine T cells (Raff 1969, McKenzie & Potter 1979). Additional murine allo-antigens have been described and correlated with functional subsets of lymphoid cells. The most useful cell surface antigens for T cell subset differentiation are the Lyt1 and Lyt2/3 alloantigens (Boyse et al. 1968). All T cells express the Lyt1 antigen but the density is variable (Mathieson et al. 1979, Ledbetter et al. 1980) with Lyt2/3$^+$ cells expressing the least Lyt1, and Lyt2/3$^-$ cells expressing the most Lyt1. In most cases the helper T cell subset expresses Lyt1 but not Lyt2/3 while cytotoxic and suppressor cells express Lyt2/3 and little Lyt1 (Cantor & Boyse 1975).

Major Histocompatibility Complex

T cell functions are influenced by a genetic region known as the major histocompatibility complex (MHC) or the H-2 complex in the mouse (Klein 1975).

The H-2 complex is located on chromosome 17 as part of linkage group IX and spans about 0.5 centimorgans of DNA. The complex is divided into four regions (Klein 1979, Klein 1981), K, I, S and D, and encodes products that can be divided into three groups or classes according to polypeptide structure.

Class I molecules, encoded by the K and D regions of the H-2 are integral membrane proteins composed of three external domains, each approximately 90 amino acids in length, a transmembrane region and a cytoplasmic domain. The third external domain is non-covalently associated with β-2 microglobulin, a small polypeptide that shows homology to the constant region domains of Ig and is not encoded in the H-2 complex (Fig. 1) (Nathenson et al. 1981,

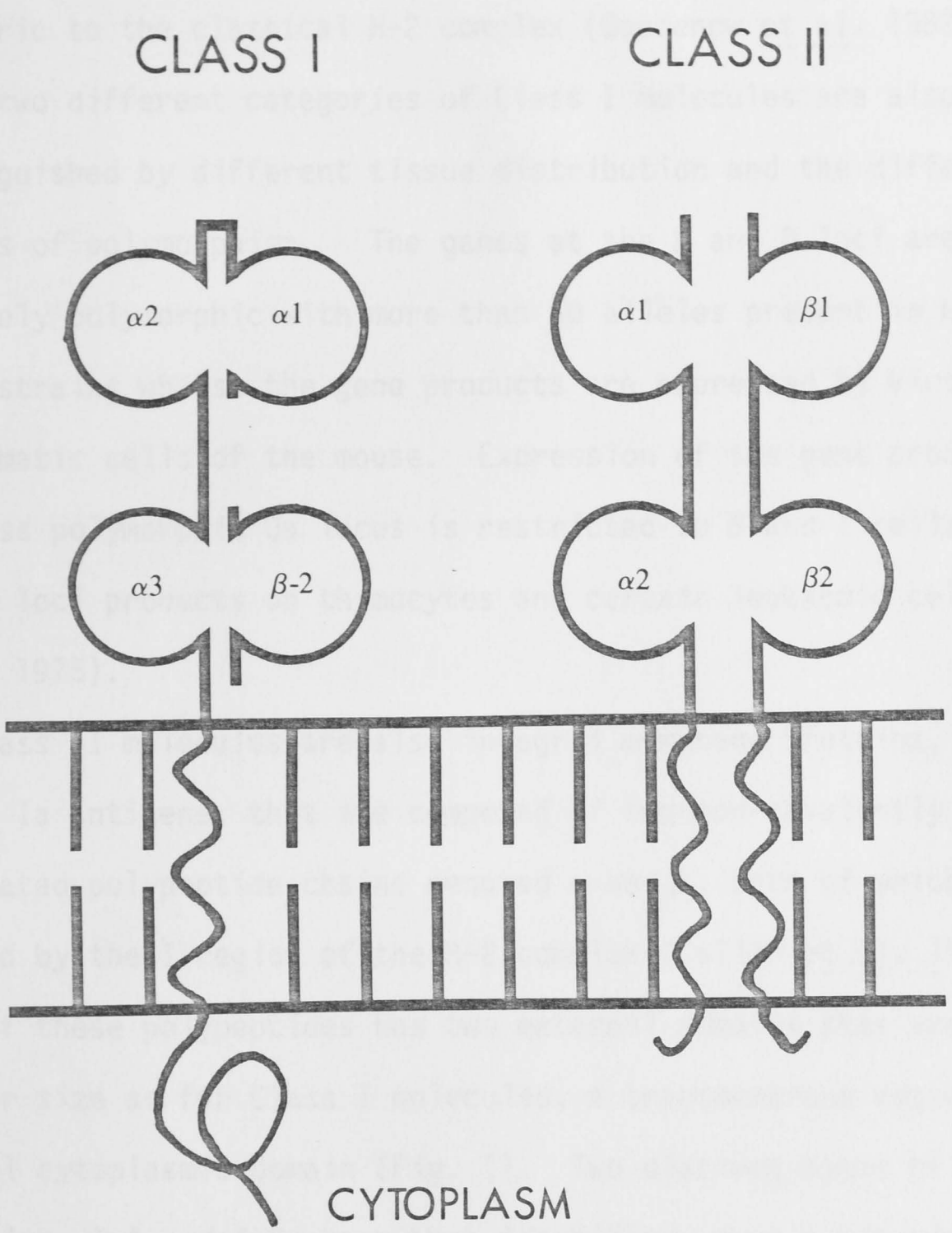

Fig. 1.
Diagram showing the domain structure of Class I and Class II, H-2 molecules and the association of β-2 micro-globulin with the Class I H-2 molecules.

Coligan et al. 1981). A second category of Class I molecules denoted Qa-1, Qa-2 and TL (which are structurally closely related to the Class I molecules or transplantation antigens encoded by the H-2K and D regions just described) are encoded by genes located telomeric to the classical H-2 complex (Goodenow et al. 1982). These two different categories of Class I molecules are also distinguished by different tissue distribution and the different degrees of polymorphism. The genes at the K and D loci are extremely polymorphic with more than 50 alleles present in known mouse strains whilst the gene products are expressed by virtually all somatic cells of the mouse. Expression of the gene products of the less polymorphic Qa locus is restricted to B and T cells with the TL loci products on thymocytes and certain leukaemic cells (Klein 1975).

Class II molecules are also integral membrane proteins, typified by the Ia antigens, that are composed of two non-covalently associated polypeptide chains denoted α and β, both of which are encoded by the I region of the H-2 complex (Cullen et al. 1976). Each of these polypeptides has two external domains that are of similar size as for Class I molecules, a transmembrane region and a small cytoplasmic domain (Fig. 1). Two distinct types of Ia molecules, I-A and I-E, have been identified, each exhibiting extensive polymorphism (Hood et al. 1983, Steinmetz & Hood 1983). Tissue distribution is limited but B cells, T cells, macrophages and dendritic cells, which include lymphoid dendritic, inter-digitating and Langerhan's cells (Steinman et al. 1979, Tew et al. 1982), are Ia positive.

Class III molecules are components of the complement pathway encoded in the S region.

K and D regions control the classical transplantation antigens responsible for the discrimination of self from non-self as defined by the rejection or acceptance of grafts (Klein 1975). These regions are also involved with cytotoxic activity of virus-specific T cells (Zinkernagel & Doherty 1974b), antihapten cytotoxicity (Shearer et al. 1975), and cytotoxic responses to minor histocompatibility antigens (Bevan 1975) and male H-Y antigens (Gordon et al. 1975).

The I region controls the induction and quantity of the immune response to polypeptide antigens (McDevitt & Chinitz 1969), antibody production to T cell-dependent antigens (Katz et al. 1975), antigen-induced T cell proliferation (Rosenthal & Shevach 1973), proliferation in mixed lymphocyte reactions (Klein 1975) and the transfer of delayed-type hypersensitivity to proteins (Miller et al. 1975, 1976) and inactivated influenza virus (Leung et al. 1980).

The involvement of the H-2 complex in T-B cell interactions was initially explained on the basis of "physiological interaction" (Katz et al. 1973a), whereby T cells possessed both antigen-specific receptors and H-2 gene complex encoded products involved in complementary recognition of somatic cells. However with the delineation of antiviral H-2 restriction it was proposed H-2 molecules were self-markers antigenically altered in a specific way by each virus or that T cells immunologically recognised both H-2 molecules and viral antigens together on the infected cell membrane, i.e., the K/D regions were involved in antigen presentation (Doherty et al. 1976). The "physiological" hypothesis was eliminated when it was shown that antiviral effector T cells in the

viral-infected F1 hybrids consisted of two different cell populations, one recognising the first parental H-2 antigens and the other recognising the second parental H-2 antigens. There was no evidence for individual F1 cells that recognised both parental antigens as predicted by "physiological interaction" (Zinkernagel & Doherty 1975).

B. LYMPHOCYTES AND HERPESVIRIDAE

Herpesviridae

The Herpesviridae, a family of ubiquitous viruses with characteristic viral particles, replicative cycles and biological features contain many examples of viruses that produce persistent or latent infection in natural or experimental hosts (Plummer 1967, Andrewes et al. 1978). Herpesviridae occur in warm and cold-blooded vertebrates and invertebrates. Each virus has its own host range which may vary considerably both in nature and in the laboratory. Transmission is usually by contact between moist mucosal surfaces although infection by blood transfusion, transplacentally, intrapartum and breast milk is possible (Ho 1982, Hamilton 1982).

Morphologically the consensus virion (120-200 nm diameter) consists of four structural components. The core is composed of a fibrillar spool on which one molecule of linear double stranded DNA (molecular weight (MW) 80-150x10^6) is wrapped. The capsid consists of 162 capsomeres arranged as an icosahedron. The globular material surrounding the capsid is known as the tegument, and the envelope is a bilayered membrane with surface projections. More than twenty structural polypeptides MW = 12,000- 222,000 have been detected whilst the lipid and carbohydrate contents have not been exactly

determined (Honess & Watson 1977).

Replication involves viral envelope adsorption to plasma membrane receptors of the host cell, fusion with the membrane and release of the viral capsid into the cell cytoplasm. Subsequently a DNA-protein complex is translocated into the nucleus where viral transcription occurs. Messenger RNA generated from these transcripts is translated in the cytoplasm. Viral DNA is replicated in the nucleus and is spooled into preformed, immature nucleocapsids. The capsids acquire an envelope by budding through the inner lamella of the nuclear membrane and virus accumulates in the cisternae of the endoplasmic reticulum and is subsequently released by transport to the cell surface through the endoplasmic reticulum (Watson 1973, Roizman et al. 1981, Mathews 1982).

Three subfamilies, Alphaherpesvirinae (Herpes simplex group), Betaherpesvirinae (Cytomegalovirus group) and the Gammaherpesvirinae (lymphoproliferative group) are recognised on the basis of genome morphology, virion antigenic properties and biological behaviour (Mathews 1982). I wish to focus on two murine models of adaptive immunity to herpesvirus infection involving (1) the natural mouse pathogen, murid herpes 1 (murine cytomegalovirus, MCMV), and (2) human herpes virus 1 (herpes simplex virus, HSV).

The in vivo Role of T lymphocytes in Herpes Virus Infection

The immune responses to herpesvirus infection can be categorised into those associated with the acute phase and those involved with the latent state and subsequent reactivation. An analysis of the mechanisms involved in latency and reactivation cannot be tackled without the necessary prerequisite of understanding the acute

immune response to viral infection. Fundamental information in other viral or bacterial infections has been derived from three approaches: (i) Quantitive studies of the course of infection *in vivo* in relation to various parameters of the normal host response such as antibody production, interferon production, macrophage activation or development of T cell-mediated immunity; (ii) Employment of animals depleted of T cells by anti-thymocyte serum treatment, neonatal thymectomy or congenitally athymic nude mice; (iii) Adoptive transfer of immune serum or lymphoid cells into infected recipients (Blanden 1974). The study of the adaptive immune response to herpesviruses has benefitted from analysis by these methods but an extra complication related to virus quantitation, that of latent infection, must be taken into account.

Unequivocal evidence that T cell dependent mechanisms were important in murine mortality following HSV inoculation via intraperitoneal (i.p.) or intragenital routes was provided by Nahmias *et al* (1969), Zisman *et al*. (1970), Oakes (1975) and Worthington *et al*. (1980a). Route of HSV inoculation and viral dose were important variables (Nahmias *et al*. 1969). Anti-lymphocyte serum treatment of mice prior to subcutaneous infection with MCMV similarly predisposed to increased mortality rates (Brody & Craighead 1974). Furthermore the mice had widely disseminated rather than limited salivary gland infection.

Neonatal thymectomy, under certain circumstances, indicated that factors dependent upon the thymus were involved in adaptive immunity to HSV. Neonatally thymectomised mice inoculated i.p. with HSV had higher mortality rates than similarly inoculated non-thymectomised mice (Worthington *et al*. 1980a). This was in contra-

distinction to Mori et al. (1967) where no significant mortality rate differences between subcutaneously HSV inoculated thymectomised and non-thymectomised mice were observed. The experimental variables of mouse and virus strains, viral doses and inoculation route, and timing of neonatal thymectomy may account for the disparate outcomes.

Experimentation with congenitally athymic nude mice also provided conflicting data. Zawatsky et al. (1979) found no significant mortality differences between nude and phenotypically normal littermates following i.p. inoculation of various HSV doses. In contradistinction, Nagafuchi et al. (1979) found limitation of skin lesions and protection from death following intracutaneous HSV inoculation was dependent upon mechanisms absent in homozygous nude athymic mice and present in heterozygous nude littermates. Homozygous nude athymic mice developed local skin lesions followed by more extensive zosteriform eruptions and died on day 18 post inoculation whilst heterozygous mice developed herpetic lesions that regressed by day 16 with no resultant deaths. Variations between two experimental protocols such as mouse strains, viral dose and strain, and inoculation route may explain the different results. However, since nude athymic mice possess activated macrophages (Zinkernagel & Blanden 1975) and macrophages are involved in protection from mortality following i.p. inoculation of HSV (Johnson 1964, Hirsch et al. 1970), the i.p. route for HSV inoculation may be inappropriate.

Quantitative estimation, by plaque forming unit (p.f.u.) assays of auricular lesions found in nude athymic BALB/c and normal BALB/c mice, revealed that the former were unable to eliminate virus from

the inoculated ear up to at least day 19, whilst all normal BALB/c mice had no detectable virus by day 14. Virus was undetectable on day 8 in some normal mice (Kapoor et al. 1982). Ganglia, spinal cord, brain and contralateral adrenal gland were sequentially infected on day 5, 8, 14 and 19 respectively in nude, athymic mice. Normal mice subjected to the same inoculation protocol possessed no viral p.f.u. in extra-auricular organs, but establishment of latent infection was not excluded. With this proviso, these data showed that factors present in normal, and absent in nude, athymic BALB/c mice, were important for limitation of HSV p.f.u. spread from the original inoculation site and were responsible for viral elimination from that site.

For MCMV, use of athymic nude mice (Starr & Allison 1977) showed these mice to be more susceptible to lethal infection with MCMV than heterozygous euthymic littermates.

Although adoptive transfers of immune serum or cells have provided information relevant to protection from or elimination of virus infection in mice (Blanden 1974), care in interpretation must be accorded to this data since many experimental variables are involved (Zinkernagel et al. 1975). In addition the adoptive transfer experiments indicate only that a particular cell or serum preparation can influence the outcome of an infection protocol. For such data to achieve biological relevance (to the recovery of the infected animal) it must be established that (i) the cell or serum preparation adoptively transferred is normally evoked during the course of an infection and (ii) the preparation is evoked at such a time during the course of the infection as to be efficacious for recovery of the infected animal.

To show the effect of transferred HSV-immune cells the functional parameters of alteration in mortality rates or virus elimination from target organs of recipients were used. Cell suspension from spleens (Ennis 1973, Oakes 1975, Rager-Zisman & Allison 1976, Nagafuchi et al. 1979, Howes et al. 1979, Worthington et al. 1980b) and lymph nodes draining subcutaneous HSV inoculation sites (Nash et al. 1980a 1981) were sources of donor HSV-immune cells but since these cells were obtained only after multiple viral inoculations of donor animals (Ennis 1973, Oakes 1975, Nagafuchi et al. 1979, Worthington et al. 1980b), it is unknown whether these donor cell populations would be evoked in the course of an acute primary HSV infection and be involved in recovery from such an infection. This necessarily mitigates conclusions drawn by the authors.

Immune spleen cells or serum removed on day 6 after a single intravenous HSV inoculation of donor mice were used to establish that adoptive transfer of immune spleen cells and not immune serum significantly increased survival rates of HSV-infected recipient mice compared to adoptive transfers of normal spleen cells or serum (Rager-Zisman & Allison 1976). The survival figures were dependent upon the number of immune spleen cells transferred, inversely related to the time of donor cell transfer after i.p. HSV inoculation of recipients and abrogated by pretreatment of donor cells with anti-Thyl antibody and complement (C'). In addition, quantitative estimation of HSV p.f.u. in brain and liver five days after HSV inoculation revealed reduction in HSV titres in both organs of immune spleen cell recipients compared with recipients that received immune serum or no transfers. Unfortunately,

interpretation of mechanisms responsible for such results is difficult since the immune cell recipients were pretreated with cyclophosphamide. From the control data that indicated increased mortality following HSV inoculation into cyclophosphamide-treated animals and the altered viral growth patterns in liver, brain and spleen, it must be assumed that the pathogenesis of HSV infection was significantly altered. Hence this study indicated that day 6 HSV immune cells and not immune serum were capable of reversing the enhanced susceptibility to lethal infection with HSV of cyclophosphamide-treated animals and initiating viral elimination from two infected organs.

Nash et al. (1980a) obtained donor cells from cervical and auricular lymph nodes (LN) of mice inoculated once into the ear with HSV 9 or 28 days previously. Recipient mice were inoculated into the ear with HSV 60 mins after intravenous immune cell transfer. Auricular viral p.f.u. were subsequently measured. Control mice that received only auricular HSV inoculation without cell transfer had higher auricular HSV p.f.u. titres on day 1, 3 and 5 post inoculation than recipients of day 9 immune cells. The recipients of day 9 or day 28 immune cells showed a trend to viral elimination by day 5 post auricular HSV inoculation whereas the control mice did not.

The temporal relationship between viral inoculation of recipients and immune cell transfer may be of critical importance for interpretation of these results. To establish that T cells have a role in recovery from HSV infection, virus elimination after initiation of infection must be shown. In this regard the above mentioned experiments hint at the possibility (lower viral

p.f.u. on day 5 versus day 3 and 1 post immune cell transfer and auricular inoculation). The lower viral titres on day 1 in immune cell recipients compared to control animals may be explained by variations in virus load generated in the animal after inoculation. The ability of immune spleen cells, mixed with HSV *in vitro*, to release lymphokines including interferons, lymphotoxins, chemotactic factor and migration inhibitory factor (Rosenberg *et al.* 1974; Rasmussen *et al.* 1974; Valle *et al.* 1975; Donnenberg *et al.* 1980) may also occur *in vivo* and alter the events at the inoculation site that lead to productive infection and replication.

The H-2 restriction pattern of these effects was also addressed (Nash *et al.* 1981) with recombinant mouse strains. Whole H-2 haplotype or H-2K and I region homology between immune spleen cell donors and HSV-infected recipients was necessary to effect a lowering of HSV p.f.u. in the ear. Homology in H-2K, D or I regions alone was not sufficient to reduce viral titres. The interpretation of this data is dependent upon the factors discussed above.

Studies with nude athymic and normal BALB/c mice reported by Kapoor *et al.* (1982) obviated the possible variations in productive infection and replication at the inoculation site, since recipient mice received immune cells 3 days after HSV inoculation. Immune cervical and auricular LN cells from donors inoculated into the ear with HSV 6 days previously, adoptively transferred into nude athymic mice, initiated elimination of HSV from the pinna and prevented HSV spread to dorsal root ganglia, spinal cord, brain and adrenal gland. Although latent infection of extra-auricular tissues was not excluded the data suggested a role for immune LN cells in elimination of acute HSV infection. Further

investigations are needed to determine the cell phenotypes involved.

For MCMV, the adoptive transfer of spleen cells, removed from donors 6 days after i.p. inoculation of MCMV, into recipients MCMV-infected i.p. 24 hours previously, produced the ability to mediate viral elimination from liver and a reduced mortality rate compared with animals recipient of normal spleen cells (Starr & Allison 1977). Adoptive transfer of day 6 serum was ineffective. Furthermore, these effects were dependent upon anti-Thyl antibody and C' treatment-sensitive cells, the number of spleen cells transferred and the viral dose of MCMV inoculated into cell recipients. These findings were confirmed and extended (Ho 1980). Spleen cell donors used day 6 to day 14 post i.p. inoculation of MCMV or immune cells generated by _in vitro_ restimulation of MCMV primed spleen cells, provided the ability to mediate viral elimination from spleen in a cell dose-dependent manner. Furthermore, H-2 restriction analysis showed a requirement for donor and recipient homology in the H-2K or H-2D region. Homology at the H-2I region did not result in viral elimination from the spleen.

The _in vitro_ Generation of Herpes Virus-specific T lymphocytes

The activity of virus-immune cells can be assessed by various detection systems that include adoptive transfer of protection from or elimination of virus infection, delayed-type hypersensitivity reactions and _in vitro_ cytotoxicity. The _in vitro_ cytotoxicity assay first used to measure cytopathic and lytic changes of tumour cells following exposure to immune spleen cells (Rosenau & Moon 1961) was subsequently extended to virus-infected target cell monolayers reacted with virus-immune lymphocytes (Speel _et al_. 1968,

Lundstedt 1969). Radioactive techniques using ^{51}Chromium labelled allogeneic (Brunner et al. 1968) and virus-infected target cells (Oldstone & Dixon 1970, Zinkernagel & Doherty 1974a,b) allowed rapid quantitative and qualitative measurement of immune lymphoid cell lytic activity.

For ectromelia and influenza viruses the cytotoxic immune cells are non-adherent to plastic, sensitive to anti-Thy1 antibody and C' treatment, insensitive to anti-mouse Ig and C' treatment and absent from rosettes with anti-mouse-Ig-coated red blood cells (i.e., negative for both Ig and Fc receptors). Direct contact between effector and target cell was required and no mandatory role for lymphokine was found (Gardner et al. 1974a,b, Yap & Ada 1977). Furthermore the immune cells were virus-specific, Class I, H-2 restricted (Blanden et al. 1975) and of the $Lyt2^{+}3^{+}$ phenotype (Pang et al. 1976). These characteristics were similarly established for other viruses (Zinkernagel & Doherty 1979). Such criteria specifically exclude other forms of cytotoxicity related to natural killer cells or antibody-dependent cell-mediated immunity (Cerrotini & Brunner 1974).

Detection of HSV-immune cells by in vitro cytotoxicity from mice exposed to primary infection with HSV has produced different results dependent upon experimental protocols used by investigators. HSV-specific cytotoxicity, dependent upon nylon-wool non-adherent, $Thy1^{+}$, Ig^{-} cells was detected in the spleen 4 to 10 days after i.p. inoculation of live HSV. Neither ultraviolet (UV) or heat-inactivated virus, nor detergent treated HSV-infected cell preparations were capable of inducing cytotoxicity (Lawman et al. 1980a). These observations are in contradistinction to others

where virus-specific cytotoxicity was not detected in LN cell populations draining primary inoculation sites of HSV in footpad or ear (Pfizenmaier et al. 1977a, Nash et al. 1980b). However, if mice were pretreated for 2 days with 80 mg/kg of cyclophosphamide prior to primary HSV footpad inoculation some cytotoxicity was detected within the draining popliteal LN cell populations (Pfizenmaier et al. 1977b): this enhancement was dependent upon cyclophosphamide dosage since the administration of 200 mg/kg resulted in no improved cytotoxicity.

For MCMV low levels of virus-specific cytotoxicity were detected on day 4 to 10 in spleen and LN cell populations following primary i.p. or intranasal virus inoculation (Quinnan et al. 1978, 1980) but reproducibility has been a problem (Ho 1980). The amount of virus-specific cytotoxicity was dependent upon viral dose. Intraperitoneal inoculation with UV-irradiated MCMV also generated virus-specific cytotoxicity in spleen cell populations but the possibility of Sendai virus contamination of MCMV stock may mitigate these results (Sethi & Brandis 1979).

No primary in vitro cytotoxicity for HSV or MCMV has been reported but this experimental protocol has also been difficult with other viruses (Blanden et al. 1977). A protocol with combined in vivo and in vitro steps produced HSV-specific cytotoxic cells (Pfizenmaier et al. 1977a). Cytotoxicity was generated if draining popliteal LN cells removed 4 to 9 days post live virus inoculation of footpads were incubated for 2 to 6 days in vitro. Levels of cytotoxicity were dependent upon viral dose inoculated into the footpad, the time of popliteal LN cell removal after virus inoculation and the length of time of in vitro incubation prior to

cytotoxicity assay. No extra viral antigen or virus was added during *in vitro* incubation and cytotoxicity was sensitive to mitomycin C treatment prior to culture. A similar system with auricular viral inoculation followed by harvest of cervical and auricular LN cells confirmed these findings (Nash *et al*. 1980b). Both groups noted the occurrence of cytotoxicity at high levels directed against uninfected self cells as well as HSV-infected cells.

Virus-primed spleen cells restimulated *in vitro* with additional virus have been utilised for HSV (Lawman *et al*. 1980a) and MCMV (Ho & Ashman 1979, Ho 1980) as convenient sources of cytotoxic reactivity that is dependent upon $Thy1^+$, nylon-wool non-adherent and Ig^- cells (Lawman *et al*. 1980a). Secondary *in vitro* HSV-specific cytotoxic T cell generation is dependent upon both the primary and restimulation events. Induction of HSV-primed spleen cells required live virus inoculation and was virus dose-dependent. Both HSV-1 and HSV-2 live virus preparations were satisfactory (Eberle *et al*. 1981). Spleen cells from 4 days to 6 months post i.p. inoculation of HSV were capable of cytotoxicity generation after *in vitro* restimulation. Antigen preparations of UV-irradiated or heat-inactivated virions, and detergent extracts of HSV-infected cells were unable to prime spleen cells.

Secondary restimulation *in vitro* of primed spleen cells was achieved with live virus, UV-inactivated HSV, gluteraldehyde inactivated HSV-infected L cells (Lawman *et al*. 1980a) and antigens from deoxycholate solubilised HSV-infected cells prepared in phosphatidyl choline and cholesterol liposomes (Lawman *et al*. 1981). Neither heat-inactivated virus nor soluble extracts of HSV-infected

L cells alone stimulated cytotoxicity. The virus-specific cytotoxicity measured after 5 days *in vitro* was also directly dependent upon the number of HSV p.f.u. added *in vitro* and inversely related to the exposure time of HSV to UV-irradiation prior to addition into spleen cell culture. Use of homologous HSV serotype in priming and restimulation events resulted in more virus-specific cytotoxicity than if heterologous preparations were used (Eberle *et al.* 1981).

Reductionist analysis of cellular requirements for secondary *in vitro* restimulation of virus primed spleen cells showed cytotoxicity was dependent upon nylon-wool adherent (Rouse & Lawman 1980) or $Thy1^+$ cells (Lawman *et al.* 1980a). With the nylon-wool non-adherent cell population cytotoxicity was restored subsequent to addition of syngeneic resident peritoneal or L cells. The amount of cytotoxicity restored was directly proportional to the numbers of peritoneal or L cells added. Viable cells were required since heated or sonicated cell extracts were ineffective. Soluble factors provided from metabolically active L cells or resident peritoneal cells may have produced these results since supernatants from 24 hour mixed lymphocyte cultures also restored the cytotoxic response of nylon-wool non-adherent spleen cells (Rouse & Lawman 1980, Schmid *et al.* 1981).

MCMV-specific cytotoxicity was generated *in vitro* by restimulation of primed spleen cells with MCMV-infected cells provided the latter were UV- or gamma-irradiated and syngeneic with the H-2 haplotype of the primed spleen cells. The cytotoxicity was dependent upon anti-Thy1 antibody and C' treatment-sensitive cells and was H-2 restricted (Ho 1980). The requirement for irradiated

cells was not explained but may relate to the immunosuppressive capability of live MCMV (Loh & Hudson 1982).

The in vitro Generation of Herpes Virus-specific Target Cells

Target cell requirements for the detection of antiviral cytotoxic T cells must involve cell surface expression of Class I MHC gene products and virus-specific antigens. Both F9 teratoma and RTL cell lines lack detectable MHC antigens at the cell surface and are not susceptible to lysis by antiviral or antiminor histocompatibility antigen-specific cytotoxic T cells respectively (Zinkernagel & Oldstone 1976, Doherty et al. 1977, Bevan & Hyman 1977). Blocking of antiviral cytotoxicity with antibody directed against Class I MHC antigens further supported the notion that Class I MHC cell surface antigens are necessary for susceptibility to lysis (Koszinowski & Ertl 1975). Manipulation of Class I encoded gene products via use of H-2 mutants indicated minimal gene and amino acid sequence alterations, not detectable by means of anti-H-2 antibodies, impaired target cell recognition by virus-specific cytotoxic T cells (Kees & Blanden 1976, Blanden et al. 1976a, Zinkernagel 1976) and provided definitive evidence of Class I gene product involvement in target-T cell interactions. Mutations clustered in the second external domain were most frequently observed to alter target cell recognition (Klein et al. 1983).

To define the viral antigen(s) necessary for target cell susceptibility to lysis by virus-specific effectors has proved more difficult. With ectromelia (Gardner et al. 1974a,b) antiviral antibodies did not block virus-specific cytotoxicity. On the other

hand anti-vaccinia and anti-vesicular stomatitis virus (VSV) antiserum from rabbits or mice inhibited virus-specific cytotoxicity (Koszinowski & Thomssen 1975, Hale et al. 1978) provided certain immunisation protocols were followed for antiserum production. Vaccinia infectious virions and early antigens from vaccinia-infected cells generated blocking antiserum whilst late antigens from vaccinia-infected cells did not induce blocking antiserum. Disrupted virions or glycoprotein G were successfully used for anti-VSV blocking serum production (Hale et al. 1978, Sethi & Brandis 1980). In the latter instance the VSV-G glycoprotein was postulated as the antigenic molecule recognised by virus-specific cytotoxic T lymphocytes but it is not possible to exclude steric hindrance as the mechanism for blocking rather than direct blockade of the virus-specific antigen-T cell interaction. Furthermore, since antigenicity as defined by cytotoxic T cells and antibody may be disparate (Blanden et al. 1976, Müllbacher et al. 1979), this approach to viral antigen detection relevant to T cell-target cell interactions may be of limited usefulness.

Minimal requirements for target cell production indicate that with some viruses no viral replication is necessary (Ada et al. 1976, Koszinowski et al. 1977, Sugamura et al. 1977, Pfizenmaier et al. 1977b, Hapel et al. 1978). Furthermore no viral genome or protein synthesis was required for Sendai (Schrader & Edelman 1977) or vaccinia provided high multiplicity of infection was employed (Hapel et al. 1978). With Sendai the generation of virus-specific targets for cytotoxicity was dependent upon the fusion activity of the virus envelope (Sugamura et al. 1978).

Cells adsorbed with live ectromelia or HSV acquired susceptibility to lysis by virus-specific cytotoxic T cells within 90 mins, provided cellular protein synthesis was not impaired. Inhibition of cellular DNA synthesis did not abrogate development of susceptibility to lysis (Ada et al. 1976, Pfizenmaier et al. 1977b). The importance of protein, and in particular glycoprotein metabolism, in target cell generation was further supported by the ability of glycosylation inhibitors tunicamycin and 2 deoxy-D-glucose to decrease susceptibility to lysis of HSV-infected target cells by virus-specific effectors, but since both inhibitors also affected susceptibility to lysis by alloreactive cytotoxic T cells a distinction between effect on viral glycoprotein versus Class I, H-2 antigen modulation was not possible (Lawman et al. 1980b, Carter et al. 1981). Glycoprotein viral antigens were implied target antigens when temperature sensitive HSV mutants, defective in viral protein synthesis, were used to generate target cells at non-permissive temperatures. Wild type HSV-specific cytotoxic T cells lysed temperature sensitive mutant infected targets much less efficiently than targets adsorbed with wild type HSV (Lawman et al. 1980b). Similar implications were drawn from VSV temperature sensitive mutants, also defective in protein synthesis (Hale et al. 1978, Zinkernagel et al. 1978), but the variables of multiplicity of infection and effects of lower temperature upon viral adsorption must be addressed prior to the resolution of the importance of viral glycoproteins as targets for virus-specific cytotoxic T cells.

Although the use of liposomes containing purified viral and H-2 antigens cannot address directly the nature of virus-specific target

antigens for cytotoxic T cells, the minimal antigenic requirements for stimulation of a virus-specific cytotoxic T cell population must, according to the clonal selection theory, be equivalent to the virus-specific target antigens. Secondary in vitro anti-VSV cytotoxic T lymphocytes could be stimulated provided the synthetic liposomes contained viral and H-2 molecules in the same vesicle and the H-2 antigen was homologous with the H-2 antigen of the virus-primed spleen cell source (Finberg et al. 1978, Loh et al. 1979). Viral antigen limited to VSV glycoprotein G was satisfactory for stimulation, but optimal cytotoxicity was generated with glycoprotein G obtained from viral preparations serologically homologous with priming virus (Hale et al. 1980).

C. LYMPHOCYTES AND LYMPHOKINES

Definition of Lymphokines and Nomenclature

The development and regulation of immune responses, and immuno-effector mechanisms, are in part dependent upon soluble mediators derived from leucocytes (Pick & Turk 1972, Rocklin et al. 1980). These non-immunoglobulin factors obtained from supernatant medium of appropriately stimulated heterogeneous cell populations derived from thymus, spleen, lymph nodes, peritoneal exudate, peripheral blood or respiratory tract (Pick & Turk 1972) were termed lymphokines (Dumonde et al. 1969). Unprimed lymphoid cells were stimulated to lymphokine production with mitogens (Wheelock 1965, Watson & Mochizuki 1980) or alloantigens (Gifford et al. 1971, Kirchner et al. 1979) whilst with bacterial (Green et al. 1969, Milstone & Waksman 1970) or viral antigens (Epstein et al. 1972,

Rassmussen et al. 1974, Rosenberg et al. 1974, Ihle et al. 1981) primed lymphoid cells were required. Spleen cells as early as day 3 to as late as 5 months post viral inoculation were suitable for lymphokine production subsequent to in vitro restimulation with homologous virus (Waldman et al. 1972, Cambridge et al. 1976, Bell et al. 1978, Donnenberg et al. 1980).

Consequent to the use of such heterogeneous cell populations and stimulation protocols, the minute quantities of soluble materials produced and lack of satisfactory physicochemical characterisation, a nomenclature based upon typical biological activities resulted. Use of different biological assays by different investigators has generated a plethora of nomenclature (Aarden et al. 1979). For convenience the known lymphokines can be divided into four major groups according to the effects mediated.

(i) Growth or maturation of haemopoietic stem cells, e.g., colony stimulating factors (CSF) for granulocyte, macrophage, eosinophil or erythroid precursors (Metcalf & Johnson 1978).

(ii) Delayed-type hypersensitivity reactions with macrophages, granulocytes and other inflammatory cells and/or the vascular endothelium (Adelman et al. 1978).

(iii) Regulation of the antibody-forming response of mature B cells (Schimpl & Wecker 1978).

(iv) Regulation of differentiation and proliferation of antigen-reactive T cells.

These categories may not be exclusive since one lymphokine source may contain multiple biological activities (Nabel et al. 1978) or one particular lymphokine may exert multiple biological effects (Gresser et al. 1979).

Nevertheless, recently two factors that modulate activation of murine lymphocytes have been distinguished on the basis of biochemical and biological criteria (Aarden et al. 1979). Both of these factors enhance the mitogenic effects of Phytohaemagglutinin-A and Concanavalin-A upon thymocytes and stimulate antigen-dependent cell-mediated and humoral immune responses in vitro. One of these factors can be obtained from human and mouse macrophages and from supernatants of macrophage tumours (Lachman et al. 1977, Mizel et al. 1978). This lymphokine was originally designated lymphocyte-activating factor (LAF) (Gery et al. 1972). The second factor is a murine spleen cell product described by Chen & DiSabato (1977) as thymocyte stimulating factor (TSF). This factor promotes and maintains long-term culture of primary T cell lines. LAF does not have this biological capability. Subsequent to the Second International Lymphokine Workshop (Ermatingen, Switzerland, May 27-31, 1979) LAF was designated Interleukin-1 (IL-1) and TSF designated Interleukin-2 (IL-2).

In vitro Generation of Lymphokines

Use of heterogeneous cell populations and stimulation protocols has also complicated characterisation of the lymphokine-producing cells, assessment of the mechanisms controlling lymphokine production and definition of the number of factors produced by one cell. Co-operative interaction between two or more cell populations appears necessary for primary in vitro stimulation of spleen cells with Concanavalin-A or alloantigen to release IL-2 (Paetkau et al. 1976, Andrus & Lafferty 1980) or γ-interferon (Newman & Sorg 1977); γ-interferon production by in vivo primed spleen cells restimulated

in vitro with homologous bacterial or viral antigens (Milstone & Waksman 1970, Sonnenfeld *et al*. 1979), and migration inhibition factor production from spleen cells primed to tumour antigens Landalfo *et al*. 1978). Depletion experiments with phenotypic antisera and C' treatment or cell separations according to physico-chemical criteria have not unequivocally resolved the issue of which cell(s) are producing lymphokines but have indicated which phenotypic groups are involved in the sequence of cellular interactions controlling lymphokine production.

However, constitutive production by T cell hybridomas or T cell clones of macrophage activating and inhibitory factors (Jones *et al*. 1981), granulocyte-macrophage stimulating and T cell proliferation factors (Nabel *et al*. 1978, Howard *et al*. 1979, Stull & Gillis 1981) suggests that for these lymphokines the T cell is the lymphokine producing cell. Similarly, the ability to stimulate with mitogens or antigens, phenotypically homogeneous cloned T cell lines (Nathan *et al*. 1981, Glasebrook *et al*. 1981), T cell hybridomas (Schrader *et al*. 1980, Katz *et al*. 1980, Kappler *et al*. 1981) and long term activated T cells (Lafferty *et al*. 1980, Marcucci *et al*. 1981) to IL-2, γ-interferon, polyclonal B cell stimulating factor, granulocyte-macrophage CSF and allogeneic effect factor production also indicates lymphokine production is a T cell function.

The control mechanisms involved in lymphokine production by non-constitutive lymphokine producers appears dependent upon the type of immunologically reactive cell populations and antigen preparations used for investigations. Resting T cells, exposed to alloantigen or mitogen, required cell to cell contact with metabolically viable accessory cells to produce lymphokine (Paetkau *et al*. 1976,

Larsson & Countinho 1979, Lafferty et al. 1980). On the other hand, Landolfo et al. (1978) showed migration inhibitory factor production by in vivo activated Moloney sarcoma virus-immune T cells was dependent upon viable accessory cells only if soluble antigens from sonicated sarcoma cells were used for stimulation. When viable Moloney sarcoma tumour cells were used, accessory cells were not required. Similarly, no viable accessory cell contact was essential for lymphokine release from homogeneous populations of alloantigen-activated T cells (Lafferty et al. 1980). Addition of Concanavalin-A alone or of metabolically inactive (UV-irradiated) allogeneic tumour cells, H-2 matched with the original priming cells stimulated IL-2 release from T cells.

Lymphoid cells have been primed to viruses, tumours, a large array of soluble proteins including tetanus and diphtheria toxoids, ovalbumins, amino acid polymers, keyhole limpet haemocyanin, and particulate antigens such as red blood cells and allogeneic spleen cells. Higher lymphokine titres were obtained by in vitro stimulation of primed lymphoid cells with homologous priming antigen, rather than with heterologous antigen (Rubin & Coons 1971, Taussig 1973, Waldman & Munro 1973) or mitogen (Green et al. 1969). With alloantigen-activated T cells, lymphokine release in vitro was dependent upon stimulation with allogeneic cells H-2 matched with the original priming cells (Eshhar et al. 1977, Lafferty et al. 1980) and the sources of allogeneic stimulator cells. Fibroblasts, thymocytes and P815 tumour cells were not as efficient stimulators as spleen cells (Eshhar et al. 1977). T cell clones and hybridomas derived from antigen-primed spleen cells released lymphokine in vitro provided restimulation occurred with both the specific

priming antigen and accessory cells that were H-2 homologous in the I region with the primed spleen cells from which the clone or hybridoma originated (Schreier & Iscove 1980, Schreier et al. 1980, Kappler et al. 1981). Hence lymphokine release is an antigen-specific T cell function stimulated by appropriate cell-associated antigen.

Lymphokines and Herpesviridae

Detection of cellular immunity to Herpesviridae by in vitro lymphokine assays has been reported with human (Rasmussen et al. 1974, Rosenberg et al. 1974), bovine (Babiuk & Rouse 1976), guinea-pig (Bell et al. 1978, Donnenberg et al. 1980) and murine (Rytel & Hooks 1977, Kirchner et al. 1978, Zawatsky et al. 1981) models.

Primed lymphoid cells restimulated in vitro with heat-activated HSV released γ-interferon (Rasmussen et al. 1974, Valle et al. 1975) and chemotactic or lymphotoxin factors (Rosenberg et al. 1974). Preparations of heat- or UV-inactivated virus, virus-infected cells and virion-neutralising antibody complexes stimulated lymphokine production from primed spleen cells (Babiuk & Rouse 1976).

Peripheral blood lymphocytes or spleen cells provided the cellular components for lymphokine production with at least two cellular populations characterised as essential for γ-interferon production; glass or plastic adherent cells and nylon-wool non-adherent, anti-Thy1-antibody and C' treatment-sensitive cells (Valle et al. 1975, Babiuk & Rouse 1976, Sonnenfeld et al. 1979).

Spleen cells removed 3 to 20 days post i.p. inoculation of live HSV released lymphokine (leucocyte migration inhibition factor or

γ-interferon) when stimulated with β-propionolactone-inactivated HSV (Kirchner et al. 1978) or soluble antigen extracts from HSV-infected cells (Bell et al. 1978, Donnenberg et al. 1980). No analyses of cellular phenotypes or antigenic requirements for stimulation of lymphokine production were reported.

CHAPTER 2

The Cytotoxic Response to Murine Cytomegalovirus.

I. Parameters *in vivo*

INTRODUCTION

The importance of T cells in recovery from acute viral infections such as the poxvirus, ectromelia (Blanden, 1974), and the myxovirus, influenza (Ada et al., 1981), has been firmly established. The role of T cells in viral diseases associated with persistent infection is less well defined. How T cell responses relate to the phenomena of chronic infection, latency and virus reactivation has not been clearly elucidated. The family Herpesviridae contains human and animal viruses that exhibit such clinical and subclinical phenomena during infection of natural hosts. Murid herpesvirus 1 or murine cytomegalovirus (MCMV), a natural mouse pathogen, is an example of such a member of this family. The murine infection also has many of the clinical and subclinical manifestations found in humans that contract infection with human herpesvirus 5 (human cytomegalovirus), making the mouse model a possible analogue of human infection.

That T cells play a role in protection from fatal infection with MCMV has been demonstrated using T cell transfer into T cell deficient nude mice (Starr & Allison, 1977). In non-fatal MCMV infections of normal mice, MCMV immune T cell transfer resulted in viral clearance from spleens (Ho, 1980). This phenomenon was Class I, H-2 restricted, implying that the cells responsible for viral elimination were anti-MCMV cytotoxic T (Tc) cells (Blanden et al., 1975), though this cannot be proven without obtaining anti-MCMV Tc clones and transferring them into

infected mice. The study of inductive requirements for such T cell responses and their regulation during primary MCMV infection are necessary prerequisites for an understanding of the latent state and how reactivation of infection may occur.

In the present studies, a method reported for human herpesvirus 1 (HSV) (Pfizenmaier et al., 1977) was adapted to MCMV. It was demonstrated that after hind footpad (f.p.) inoculation of MCMV there was an increase in viable cell numbers within the draining popliteal lymph nodes (LN). Upon direct in vitro assay of these LN cells no anti-MCMV cytotoxic activity was detected, but anti-MCMV cytotoxicity was generated after several days in culture of the LN cells.

METHODS

Mice. BALB/c, CBA/H and WEHI-3, 6-12 weeks of age were obtained from the Animal Breeding Establishment of the John Curtin School of Medical Research.

Viruses. The Smith strain of MCMV was obtained from Dr. G. Shellam, University of Western Australia. Salivary gland virus stock was prepared by intraperitoneal (i.p.) inoculation of $10^{4.3}$ plaque-forming units (p.f.u.)/0.2 ml into 4-week-old BALB/c female mice. At day 17 post inoculation, salivary glands were harvested and a 50% wt/vol homogenate prepared in Dulbecco's Modified Eagle's Medium (Cat. No. H16, GIBCO, Grand Island, N.Y., U.S.A.), supplemented with 5% foetal calf serum (FCS) (Flow Labs, Stanmore, N.S.W., Australia), 200 ug/ml Streptomycin, 200 U/ml Penicillin G and 125 ug/ml Neomycin sulphate. (The complete medium is referred to hereafter as DMEM.) Aliquots were stored at -70°C. For dilution of virus stock 0.2M borate-buffered gelatin saline (pH 7.2-7.4) was used. Normal salivary gland homogenate was prepared from normal 6-week-old female BALB/c mice using the same methods and diluents.

Target cell culture. Mouse embryo fibroblast (MEF) cultures were prepared from 16-18 day BALB/c or CBA/H embryos by trypsin dispersion and grown in DMEM. $75cm^2$ tissue culture flasks (Nunc, Roskilde, Denmark) were seeded at $10^{6.3}$ cells/flask and incubated at 37°C for 4 days in 10% CO_2, 7% O_2 and 83% N_2 (special

gas) (Commonwealth Industrial Gases, Alexandria, N.S.W., Australia), after which time the cells were either subcultured immediately or kept at room temperature for up to 2 weeks prior to subculture. Conditions of subculture were the same as described for the primary cultures, except that these secondary MEF cells were always used after 4 days at 37°C as the source for tertiary MEF target cells in cytotoxicity assays.

Generation of anti-viral effector cells. Anti-MCMV effector cells were generated as follows: Seven days after inoculation of 40 ul of MCMV stock ($10^{4.6}$ p.f.u.) into the hind f.p., popliteal LN were collected in Hank's balanced salt solution (HBSS), and passed through a stainless steel mesh to produce a single-cell suspension. The LN cells were cultured in Eagle's Minimal Essential Medium (EMEM) (Cat. No. F-15, GIBCO, Grand Island, N.Y., U.S.A.) supplemented with 10% FCS, 200 ug/ml Streptomycin, 100 U/ml Penicillin G and 125 ug/ml Neomycin sulphate (antibiotics), 10^{-4}M 2-mercaptoethanol (2ME) and usually 3% vol/vol Concanavalin-A activated cell supernatant. The LN cells were cultured at $10^{5.8}$ cells/ml in 5 ml Costar wells (Cat. No. 3512, Costar, Cambridge, Mass., U.S.A.) and incubated at 34°C in atmosphere containing special gas.

Removal of immunoglobulin-positive (Ig^+) cells. Popliteal LN cells were depleted of Ig^+ cells by a one-step procedure (Parish et al., 1974). Briefly, rosettes containing Ig^+ cells were obtained by reacting LN cells with sensitized sheep red blood cells coated with sheep anti-mouse immunoglobulin.

The rosetted cells were separated from non-rosetting lymphocytes by sedimentation on Ficoll-Hypaque.

Cytotoxicity assay. For MEF target cells, 96-well flat-bottomed microtitre trays (Nunc, Roskilde, Denmark) were seeded with tertiary MEF at $10^{4.0}$ cells/0.2ml/well 3 days prior to assay. After 2 days in culture, each well was drained and the cells were infected with 25 ul volume of MCMV stock in DMEM at 37°C in special gas mixture, providing a multiplicity of infection (MOI) of 5 p.f.u./cell. Subsequent to viral adsorption for 1 hr 6 uCi of $Na_2[^{51}Cr]O_4$ (^{51}Cr) (Amersham Int. Ltd., Amersham, U.K.) in 150 ul of DMEM was added to each well. At 16 hrs incubation, the targets were washed twice with DMEM and 100 ul of effector cells added immediately. Triplicate cultures were set up for each dilution of effector cells and incubated for 10 hrs at 37°C in a humidified atmosphere containing special gas.

^{51}Cr release from the targets assayed by gamma emissions was measured and lysis calculated using the following formula:

$$\%\ \text{lysis of infected or uninfected targets} = \frac{^{51}\text{Cr counts in presence of effector cells} - ^{51}\text{Cr counts released in medium}}{^{51}\text{Cr counts water-lysed targets} - ^{51}\text{Cr counts released in medium}} \times 100\%$$

% specific lysis = % lysis infected targets - % lysis uninfected targets.

Preparation of Concanavalin-A activated spleen cell supernatant (CSS). CSS was prepared using a modification of a published method (Pick & Kotkes, 1977). Spleen cells from WEHI-3 mice were cultured in serum-free EMEM with 10^{-4}M 2ME and Concanavalin-A (Pharmacia Fine Chemicals A.B., Uppsala, Sweden) at a final concentration of 5 ug/ml. All CSS preparation was carried out in the absence of FCS. Cultures were set up in 75cm^2 plastic tissue culture flasks and maintained at 37^{o}C in an atmosphere of special gas for 2 hrs. The cell monolayer was then washed gently 3 times with warm HBSS and replenished with 30 ml fresh serum-free EMEM containing 10^{-4}M 2ME. The flasks were reincubated at 37^{o}C in a humidified atmosphere of special gas for 17 hrs. The supernatant was harvested, centrifuged to remove any cells and concentrated 10-fold on an Amicon PM-10 membrane (Amicon, Danvers, Mass., U.S.A.). The concentrated CSS preparation was sterilized by filtration and stored at -20^{o}C. The preparation was added to cultures at a concentration of 3% volume/volume.

Plaque assay for MCMV. A modification of an MCMV plaque assay (Gould & Mims, 1978) was utilised. Briefly, secondary BALB/c MEF cells were seeded into 24 well Linbro tissue culture trays (Cat. No. 76-033-05, Flow Labs, Maclean, Virginia, U.S.A.) at $10^{5.3}$ cells/well/2 ml in DMEM and incubated at 37^{o}C in special gas. After 24 hrs the medium was aspirated and 50 ul volumes of virus suspension in DMEM was added to the cell monolayer. Adsorption was carried out at 37^{o}C for 60 mins after which time 2 ml of a 0.5% carboxymethylcellulose in DMEM overlay was added and incubation continued for 5 days at 37^{o}C in special gas. The

overlay was then aspirated and a staining solution composed of 0.005% crystal violet in Formol Saline added for 30 mins. Plaque counting was performed using a binocular microscope with 10x magnification. All virus suspensions were sonicated for 30 seconds using a Branson B12 Sonifier (Branson Sonic Power Co., Danbury, Conn., U.S.A.) at 50 c.p.s. prior to plaque assay.

RESULTS

The experimental program investigated modifications of the method reported by Pfizenmaier et al. (1977), to determine whether anti-MCMV Tc cell responses could be generated in cells from popliteal LN draining the site of MCMV inoculation if they were removed from the animal and maintained in vitro. The initial experiments were designed to determine the optimal time intervals in vivo and in vitro for anti-MCMV cytotoxic cell generation. For each in vivo and in vitro time point combination, 3 BALB/c female mice were inoculated into both hind f.p. with $10^{4.6}$ p.f.u. of MCMV. The six popliteal LN were removed on day 0, 2, 4, 6 or 8 post inoculation. The LN cells were pooled, cultured for 0, 2, 4, 6 or 8 days without addition of viral antigen and then assayed for cytotoxic activity against MCMV-infected and uninfected control BALB/c MEF target cells. The assays for a particular time point in vivo coupled with variable times in vitro were performed as one experiment, e.g., the assays for day 6 in vivo with days 0, 2, 4, 6 or 8 in vitro were performed on one day.

Cytotoxicity against uninfected control MEF target cells was maximal in LN cell populations harvested 4 days after viral inoculation and cultured for 2-6 days (Fig. 1). Lysis of MCMV-infected MEF targets over and above the lysis of uninfected controls required minimum intervals of 4 days in vivo post inoculation of virus plus 2 days in vitro prior to the cytotoxicity assay (Fig. 2). Maximum antiviral cytotoxic responses required 6-8 days in vivo post inoculation of MCMV plus at least 4 days of

culture. The culture of LN cells from animals at day 0 and day 2 post f.p. inoculation of MCMV did not produce cytotoxic activity even after 6-8 days in vitro (Fig. 2).

Infectious MCMV titres in lymph nodes on different days after f.p. inoculation

Twenty BALB/c female mice, 6-12 weeks of age, were inoculated into both hind f.p. with $10^{4.6}$ p.f.u. MCMV. At 1 hr, 3 days, 6 days or 9 days after inoculation, draining popliteal and iliac LN were removed from 5 animals into DMEM and stored at -70°C until assayed for p.f.u. On the day of assay, the LN were thawed and sonicated for 30 secs. The results (Table 1) showed that infectious virus could be detected in draining popliteal LN of all 5 mice on day 3, in 1/5 LN on day 6, and in no LN on day 9. The iliac LN revealed a similar temporal profile for virus titres except that not all the iliac LN were infected on day 3.

Kinetics of anti-MCMV cytotoxic activity with different viral doses

Four groups of 15 BALB/c female mice were inoculated into both hind f.p. with $10^{6.1}$, $10^{5.6}$, $10^{4.6}$ or $10^{2.6}$ p.f.u. of MCMV. On days 0, 2, 4, 6 and 8 post inoculation the draining popliteal LN from 3 mice of each group were collected and single cell suspensions were prepared. The viable cells were counted, cultured for 4 days at $10^{5.8}$ cells/ml and then assayed for cytotoxic activity against MCMV-infected and uninfected BALB/c MEF. The f.p. inoculations

for a particular viral dose were staggered at 2-day intervals such that LN harvest, cell culture and subsequent cytotoxicity assays could be performed at the one time. The assays for the different viral doses were performed on different days for logistic reasons, hence the absolute values for % lysis induced by different viral doses are not comparable; only the temporal profiles can be compared.

The results (Fig. 3) showed the development of anti-MCMV cytotoxic activity in all cell pools with maximum activity at days 6-10 post MCMV inoculation. Cytotoxic activity did not relate to the viable cell numbers in the popliteal LN at the time of harvest (Fig. 4). Cytotoxic activity against uninfected targets was maximal on day 4 for all viral doses (data not shown).

Because cell numbers in the popliteal LN did not correlate with the anti-MCMV cytotoxic activity generated in vitro from these cells, it was of interest to determine if the salivary gland homogenate in the stock virus preparations rather than MCMV caused the changes in LN cell numbers. Therefore on days 0, 2, 4, 6 and 8 after bilateral hind f.p. inoculation of $10^{4.6}$ p.f.u. of MCMV in 40 ul of diluted, infected salivary gland homogenate, both popliteal LN of 3 BALB/c mice were obtained, cells pooled and viable cell counts performed. All mice were injected on the same day with the subsequent LN collections staggered 2 days apart. A similar protocol was used for control mice that were injected with normal salivary gland homogenate.

Cell numbers in the popliteal LN of MCMV-infected mice increased to a maximum at 6-8 days post viral inoculation (Fig. 5). No significant cellular increase occurred after normal salivary

gland injection. A subsequent experiment that utilized a similar sampling protocol was performed on days 0, 1, 2, 5, 6, 8, 10, 14 and 19 after bilateral hind f.p. inoculation. There was an increase in cell numbers in the popliteal LN of MCMV-infected mice with a peak in cell numbers at 6-8 days post MCMV inoculation and a decrease to normal cell numbers by day 19 (data not shown). Similar responses to MCMV were obtained using CBA/H mice (data not shown).

The data in Fig. 3 suggested that viral doses from $10^{2.6}$ p.f.u. up to $10^{6.1}$ p.f.u. stimulated anti-MCMV cytotoxic responses provided 6-10 days elapsed between viral inoculation and LN cell harvest followed by 4 days in culture. On this basis, 7 days post-inoculation *in vivo* and 4 days *in vitro* were selected as the standard interval for further studies. An example of such an experiment using different viral doses is shown in Table 2. Viral doses of $10^{2.6}$ or $10^{4.6}$ p.f.u. were then used in further experiments using this protocol to be described in this and the following paper (Sinickas *et al.*, 1984, submitted).

Direct lymph node assay

Tests were conducted to ascertain if anti-MCMV cytotoxic activity could be detected in draining popliteal LN of MCMV-infected mice without the 4 days in culture. Groups of 3 BALB/c mice were inoculated with $10^{4.6}$ p.f.u. into both hind f.p. at intervals of 2-5 days. On days 0, 2, 4, 6, 8, 10, 12, 17 and 19 post inoculation, the six popliteal LN of each group were harvested and a 1/12 aliquot of each group's pooled LN cells were assayed for cytotoxic

activity on MCMV-infected and uninfected BALB/c MEF targets. Positive control anti-MCMV cytotoxic cells (to ascertain MCMV-infected MEF target sensitivity to lysis) were obtained as described above by harvesting LN on day 7 post inoculation of MCMV, pooling LN cells and culturing for 4 days.

To test the possibility that infected B cells in the LN cell pool were acting as cold competitor targets in the cytotoxicity assay, a cell suspension was prepared from another group of six LN at day 7 post MCMV inoculation and the cell population was depleted of Ig^+ cells prior to cytotoxic assay of a 1/12 aliquot of remaining cells.

Anti-MCMV cytotoxicity was not detected by direct assay of popliteal LN cell pools at any time post infection (data not shown). The removal from the day 7 cell pool of Ig^+ cells did not unmask the presence of cytotoxic cells (Table 3).

DISCUSSION

This paper describes experimental procedures leading to the generation of murine cytotoxic cells that lyse MCMV-infected MEF target cells. The system chosen for study was similar to that described for HSV (Pfizenmaier *et al.*, 1977) and utilised bilateral hind f.p. inoculation of salivary gland MCMV, and harvest of draining popliteal LN followed by culture of single-cell suspensions. This and the accompanying paper (Sinickas *et al.*, 1984, submitted) report the detailed characterisation of the individual steps of the experimental protocol in order to study the kinetics, specificity and other relevant parameters of the cytotoxic response. As with HSV, the expression of cytotoxic potential against MCMV-infected MEF target cells was dependent upon a further period of time *in vitro* after the LN cells were removed from the mouse. Four days *in vitro* was consistently sufficient for this purpose.

MCMV doses from $10^{4.6}$ up to $10^{6.1}$ p.f.u. resulted in cells with anti-MCMV cytotoxic potential being present in the popliteal node from 4 to 10 days after infection with maximal levels at about 8 days. After a dose of $10^{2.6}$ p.f.u., potential cytotoxicity did not appear until 6 days after infection and then persisted at maximal levels until day 10. Time points after day 10 were not studied but it would be of interest to investigate the temporal decline of cytotoxicity. MCMV was detected in the draining popliteal LN ($>10^{3.0}$ p.f.u.) 3 days after inoculation of $10^{4.6}$ p.f.u. into the f.p., was barely detectable on day 6, and was

undetectable by day 9. These results suggest that several days may be needed for MCMV to multiply in some cells of the tissues of the foot and/or of the LN, resulting in display of MCMV-dependent cell surface antigens recognised by precursors of cytotoxic lymphocytes. However, even the lowest dose of virus used ($10^{2.6}$ p.f.u.) was sufficient to stimulate an anti-MCMV response.

Numbers of viable cells in the draining popliteal LN increased after MCMV inoculation in the f.p. and the extent of increase was inversely related to the dose of virus. Thus, up to 10-fold increases in cell numbers were found 6-8 days after $10^{2.6}$ or $10^{4.6}$ p.f.u. of MCMV, but $10^{6.1}$ p.f.u. caused only an initial increase in cell numbers that was not sustained beyond the second day after inoculation. These data imply that high doses of MCMV can interfere with processes ~~what~~ which would lead to increases in LN cell numbers such as local cell proliferation or migration of cells into the LN via the blood or afferent lymph, but we have not evaluated this aspect further. The majority of the cells contributing to the increase in cell numbers were not directly relevant to the anti-MCMV cytotoxic response since the anti-MCMV cytotoxic potential was unrelated to the numbers of cells in the LN at time of removal for culture.

A feature of the popliteal LN cell response to MCMV infection was that from 4 days after infection significant cytotoxicity was expressed against uninfected self MEF target cells. This effect was most pronounced with LN cells taken 4 days after infection and cultured for 2-6 days, but was also present in uncultured LN cells taken from 4-8 days after infection. Autoreactivity has been reported previously as a feature of antiviral responses *in vivo*

(Blanden & Gardner, 1976) and *in vitro* (Gardner & Blanden, 1976) and has been attributed to either cytotoxic T cells (Blanden & Gardner, 1976, Gardner & Blanden, 1976) or natural killer cells (Welsh & Zinkernagel, 1977). It is a possible complication in the interpretation of recent work involving limiting dilution to isolate clones of Tc cells responding to MCMV infection (Reddehase *et al.*, 1984). Since the clones were not divided and assayed on both MCMV-infected self targets and uninfected self targets, it was not determined with certainty which clones were MCMV-specific and which were autoreactive.

Although autoreactive cytotoxic activity was present in popliteal LN cells taken directly from MCMV-infected mice 4-8 days after infection, MCMV-specific cytotoxicity over and above autoreactivity was not directly detectable at any time up to 19 days after infection. The possibility that infected cell populations within the LN pool were acting as cold competitor targets during the direct *in vitro* cytotoxicity assay, thereby decreasing the sensitivity of the assay for MCMV-specific cytotoxic cell detection, was tested by removal of the largest cell pool likely to be MCMV-infected (Ig^+ cells, Olding *et al.*, 1975) prior to *in vitro* cytotoxicity testing. No anti-MCMV cytotoxic activity was obtained from the draining popliteal LN on day 7 post inoculation of $10^{4.6}$ p.f.u. MCMV. This does not exclude the presence of other infected cells in the non-Ig^+ cell fraction, since we made no assessment of viral antigen expression within this cell pool. Nevertheless, since Ig^+ cells comprise approximately 50% of the LN cell pool, a major possible infected cell source was removed without effect.

The inability to detect direct cytotoxicity agrees with difficulties reported by other workers for MCMV (Ho, 1980) and HSV (Pfizenmaier et al., 1977), but is in contradistinction to others who were able to detect cytotoxicity to MCMV from two lymphoid populations, spleen and cervical LN after intraperitoneal and intranasal routes of inoculation respectively (Quinnan et al., 1978, 1980). The discrepancy may be accounted for by technical differences in target cell and virus preparations and manipulations of variables such as mouse ages, route of inoculation and viral dose. All of the latter are important parameters for mouse survival against MCMV infection (Mannini & Medearis, 1961), and hence may be important factors affecting the generation of effector cells responsible for viral elimination.

In our hands 2-4 days in vitro were always required for anti-MCMV cytotoxicity activity to develop in popliteal LN cells taken from the mouse 6-10 days after infection. The presence of CSS in the culture medium was necessary for the consistent expression of this anti-MCMV cytotoxic potential, thus raising the possibility that soluble factors which are necessary for the proliferation and/or differentiation of anti-MCMV cytotoxic cells are limiting or are insufficient to overcome some suppressor mechanism in vivo. The lack of a detectable cytotoxic response in vivo is an important feature of the immunobiology of herpesvirus infections, since many other virus groups have been shown to provoke primary antiviral Tc cell responses in vivo within 4-8 days (Blanden & Gardner, 1976, Yap & Ada, 1977). Therefore, we have undertaken a thorough reductionist approach to the separate steps of the experimental protocol required to generate and assay anti-MCMV cytotoxic cells,

beginning with the *in vivo* parameters studies here. Future reports will deal with the *in vitro* parameters, characterization of effector cells, and factors affecting the efficiency of their recognition of MCMV-infected target cells.

SUMMARY

Combined *in vivo-in vitro* protocols for the generation of anti-MCMV cytotoxic responses were investigated using BALB/c mice and syngeneic MEF target cells. Injections of doses of MCMV from $10^{2.6}$ up to $10^{6.1}$ p.f.u. into the hind f.p., harvest of draining popliteal LN cells after 6-8 days, followed by 4 days culture of these cells gave similar and optimal cytotoxic activity against MCMV-infected target cells. After injection of $10^{4.6}$ p.f.u. into the hind f.p., MCMV was detectable at about 10^{3}-10^{4} p.f.u. per popliteal node after 3 days, but became undetectable in most animals by 6 days. Cell numbers in the draining popliteal LN increased following MCMV inoculation into the hind f.p., but the extent of the increase was inversely related to virus dose and bore no relationship to the anti-MCMV cytotoxic potential of the cells. LN cells applied directly to target cells upon harvest from MCMV-infected mice never gave detectable anti-MCMV cytotoxic activity at 2, 4, 6, 8, 10, 12, 17 or 19 days post infection, but lysis of uninfected syngeneic targets was obtained 4-8 days post infection.

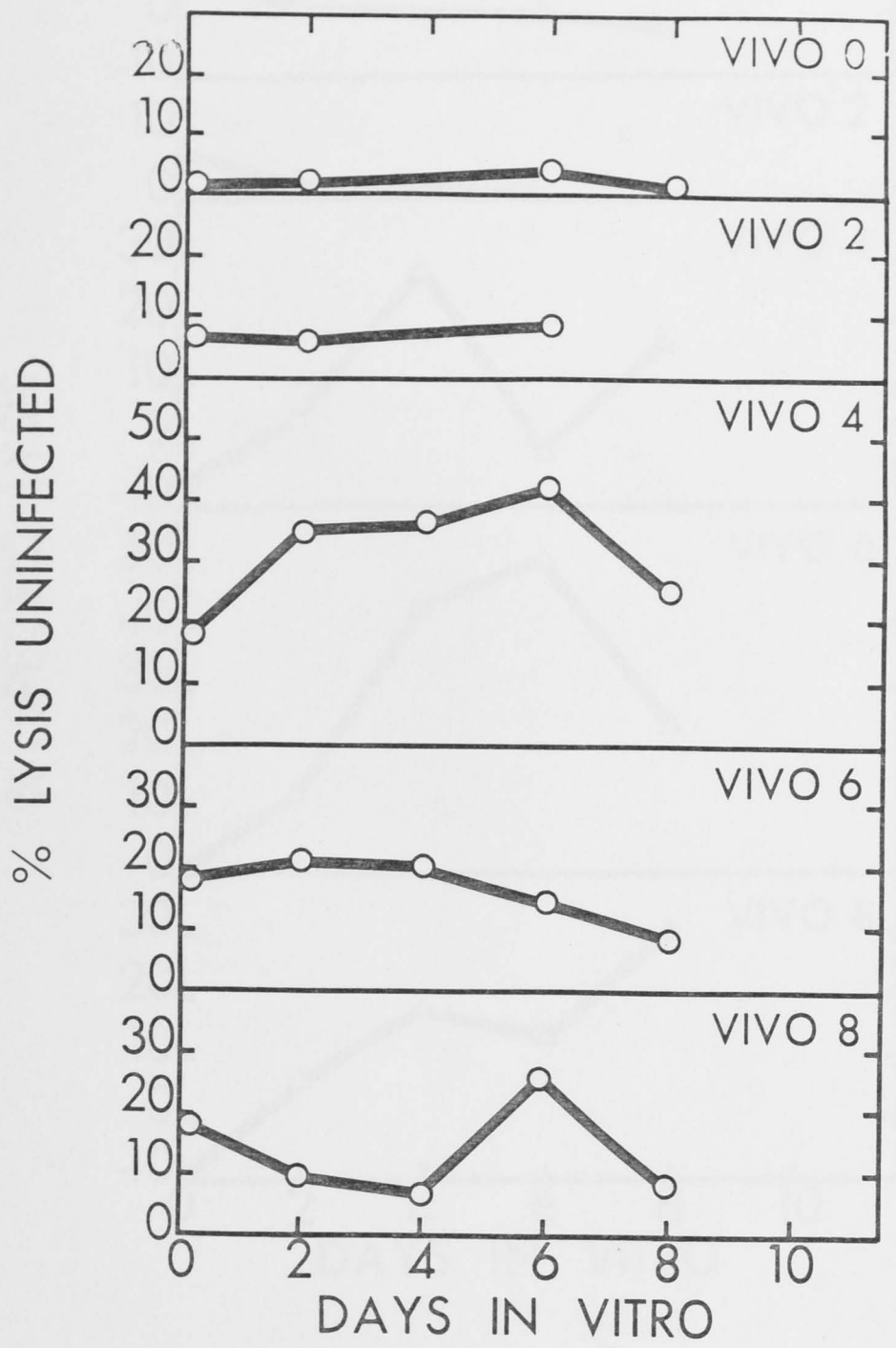

Fig. 1.

Lysis of uninfected BALB/c MEF target cells by effector cells generated from 6 BALB/c popliteal LN removed on various days (indicated by vivo on figure) after f.p. inoculation of $10^{4.6}$ p.f.u. of MCMV and cultured as single-cell suspensions (indicated by days *in vitro*) for various times. The % lysis values are the means of three replicates obtained by addition of a 1/12 aliquot of cultured cells from 6 popliteal LN onto targets. The standard errors of the mean were <4% and are not shown.

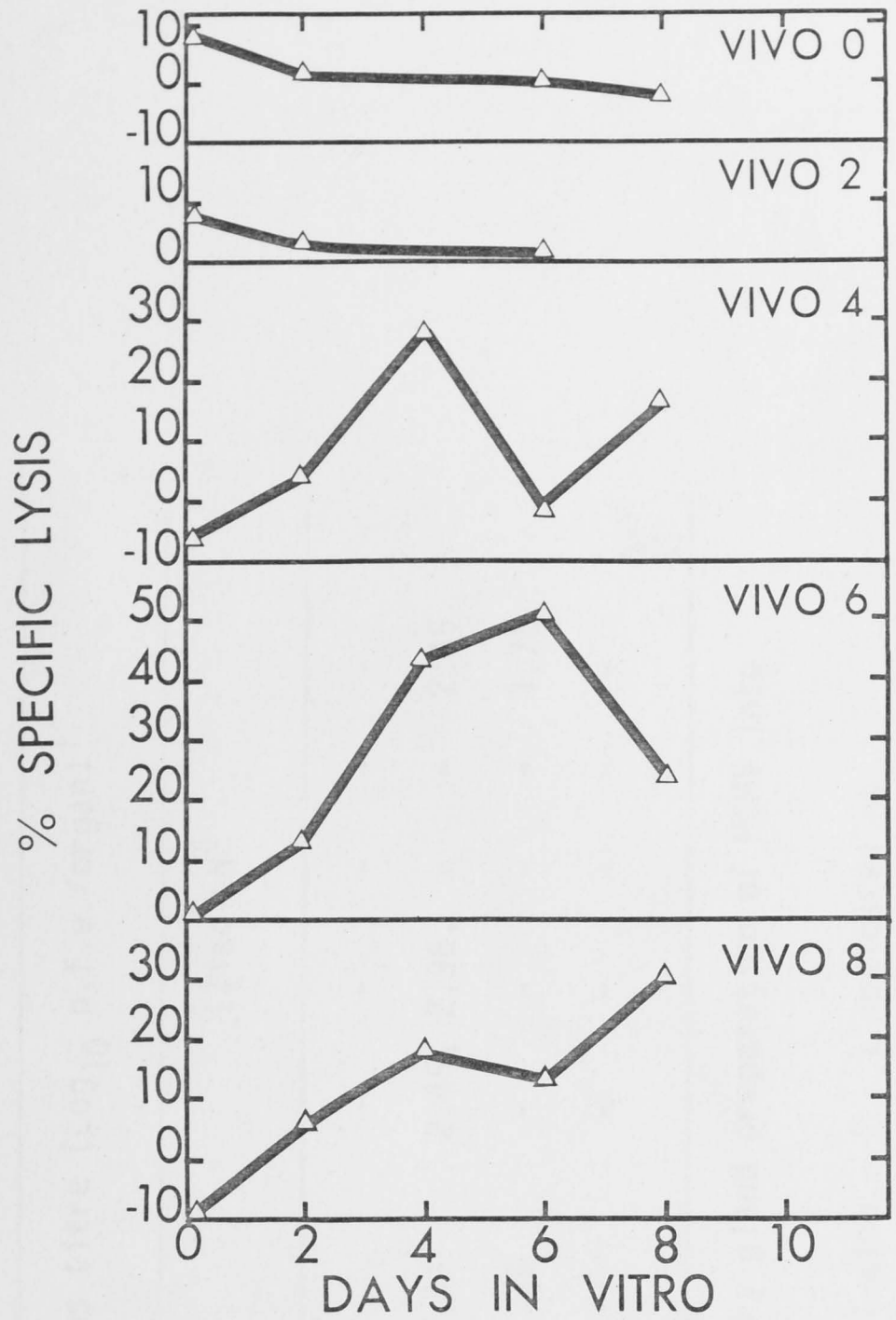

Fig 2.

Specific lysis of MCMV-infected BALB/c MEF target cells by effector cells generated as described in Fig. 1. Percent lysis values were obtained by addition of a 1/12 aliquot of cultured cells from 6 popliteal LN onto MCMV-infected and uninfected target cells. The % specific lysis values are the result of subtraction of the lysis obtained on uninfected targets from lysis detected on MCMV-infected targets. Each point represents the mean of 3 replicates. The standard errors of the mean were <4% and are not shown.

TABLE 1

Infectious MCMV titres in lymph nodes at different times after infection*

Time after infection (days)	Infectious virus titre (Log_{10} p.f.u./organ)†									
	Popliteal LN‡					Iliac LN§				
0.04	-	-	-	-	-	-	-	-	-	-
3	3.54,	3.38,	3.51,	3.0,	3.51	2.45,	2.96,	-	-	2.45
6	-	-	-	-	1.78	-	-	-	-	1.78
9	-	-	-	-	-	-	-	-	-	-

* Mice were infected with $10^{4.6}$ p.f.u. salivary gland preparation of MCMV into both hind footpads.

† Viral p.f.u. values are shown for individual mice. (-) indicates no infectious virus detected.

‡ Two popliteal LN/animal are treated as one lymphoid organ.

§ Four iliac LN/animal are treated as one lymphoid organ.

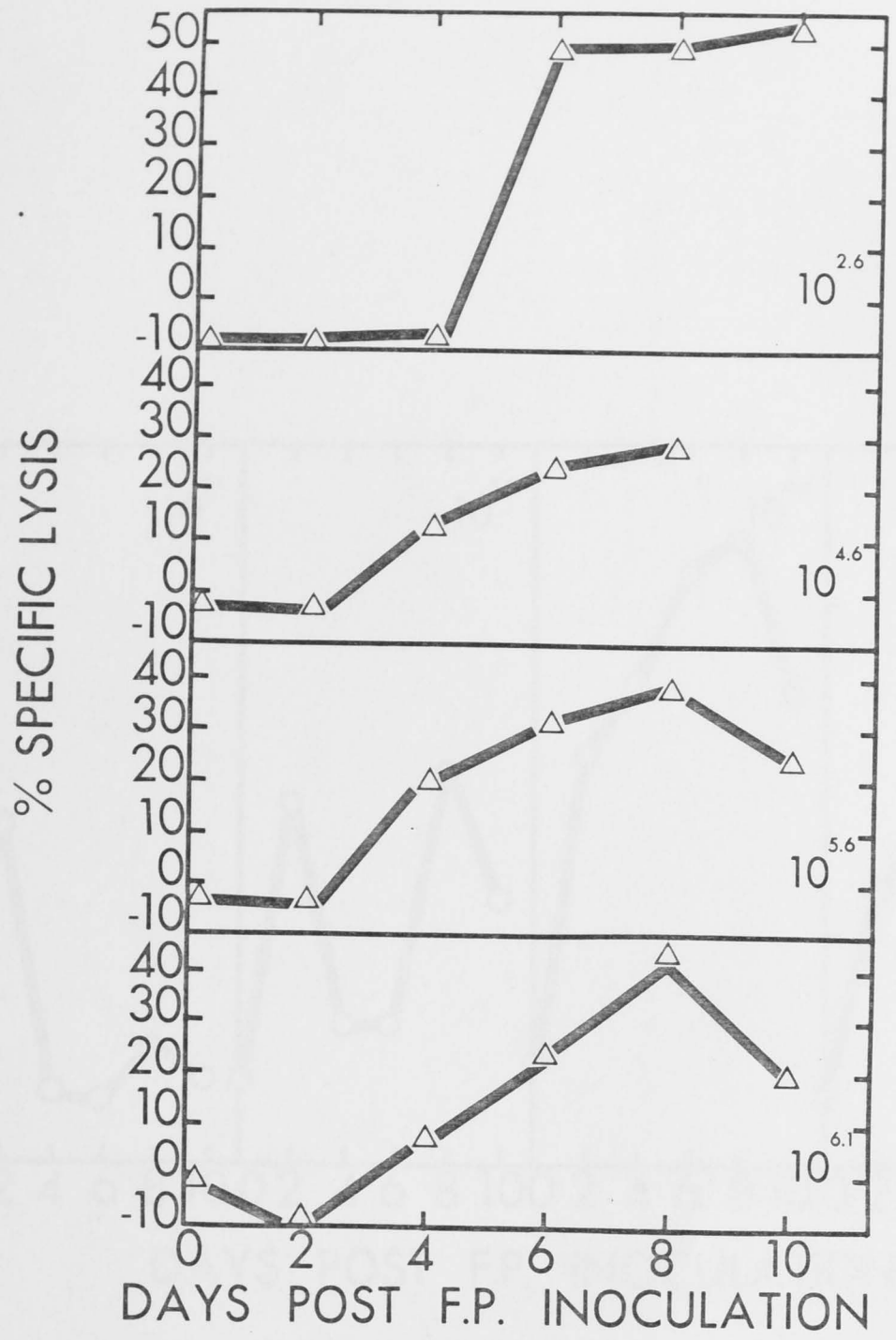

Fig. 3.

Kinetics of anti-MCMV cytotoxic activity with different viral doses. Effector cells were generated from 6 BALB/c popliteal LN removed at various days after f.p. inoculation of $10^{6.1}$, $10^{5.6}$, $10^{4.6}$ or $10^{2.6}$ p.f.u. MCMV and cultured as single-cell suspensions for 4 days. The % specific lysis values were obtained as described in Fig. 2. The standard errors of the mean were <4% and are not shown.

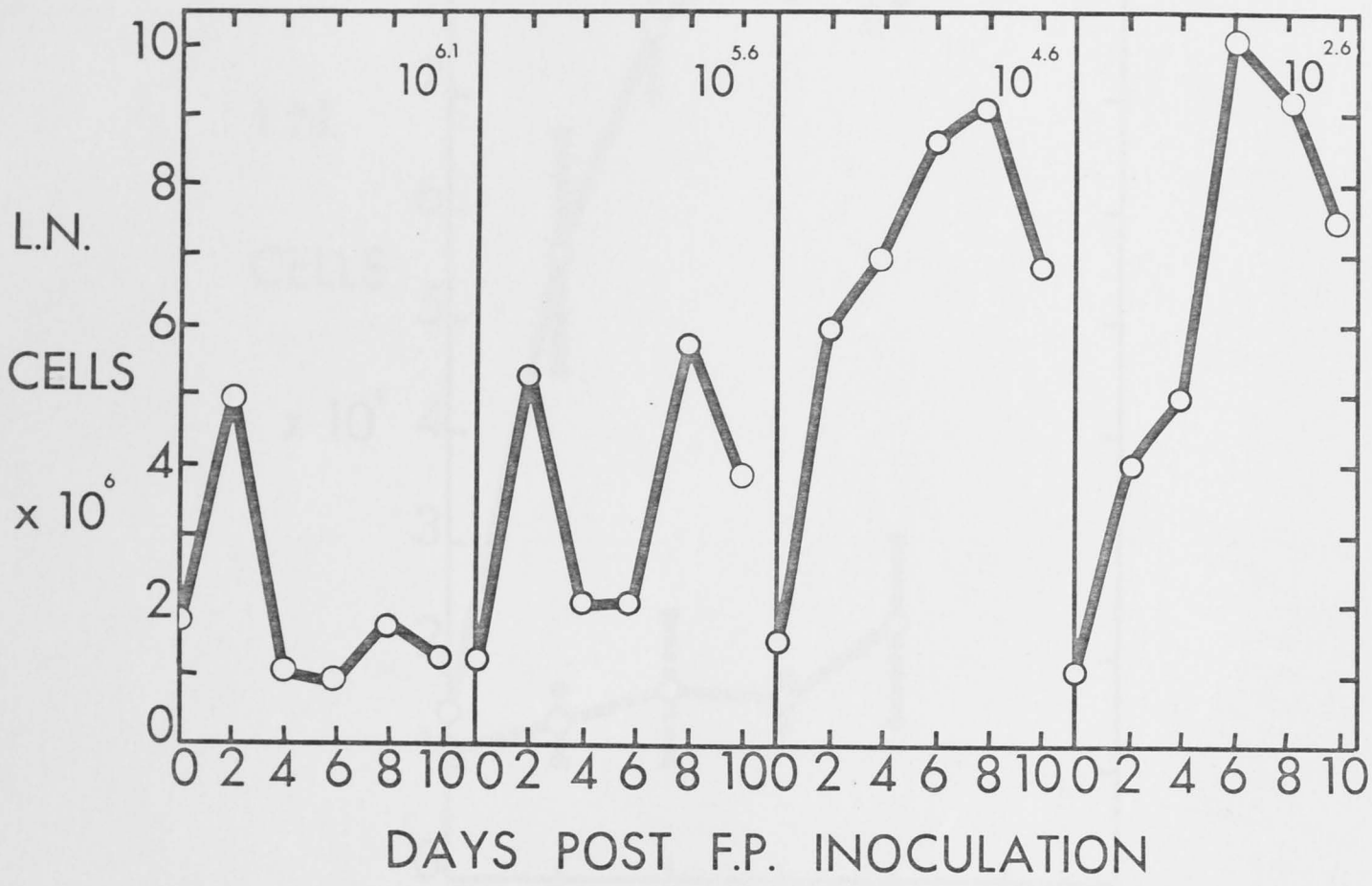

Fig. 4.

Viable cell numbers in draining popliteal LN after hind f.p. inoculation of different doses of MCMV. Each point represents the mean numbers of viable cells per LN obtained from six pooled popliteal LN at the indicated times post inoculation of the viral doses shown (p.f.u.).

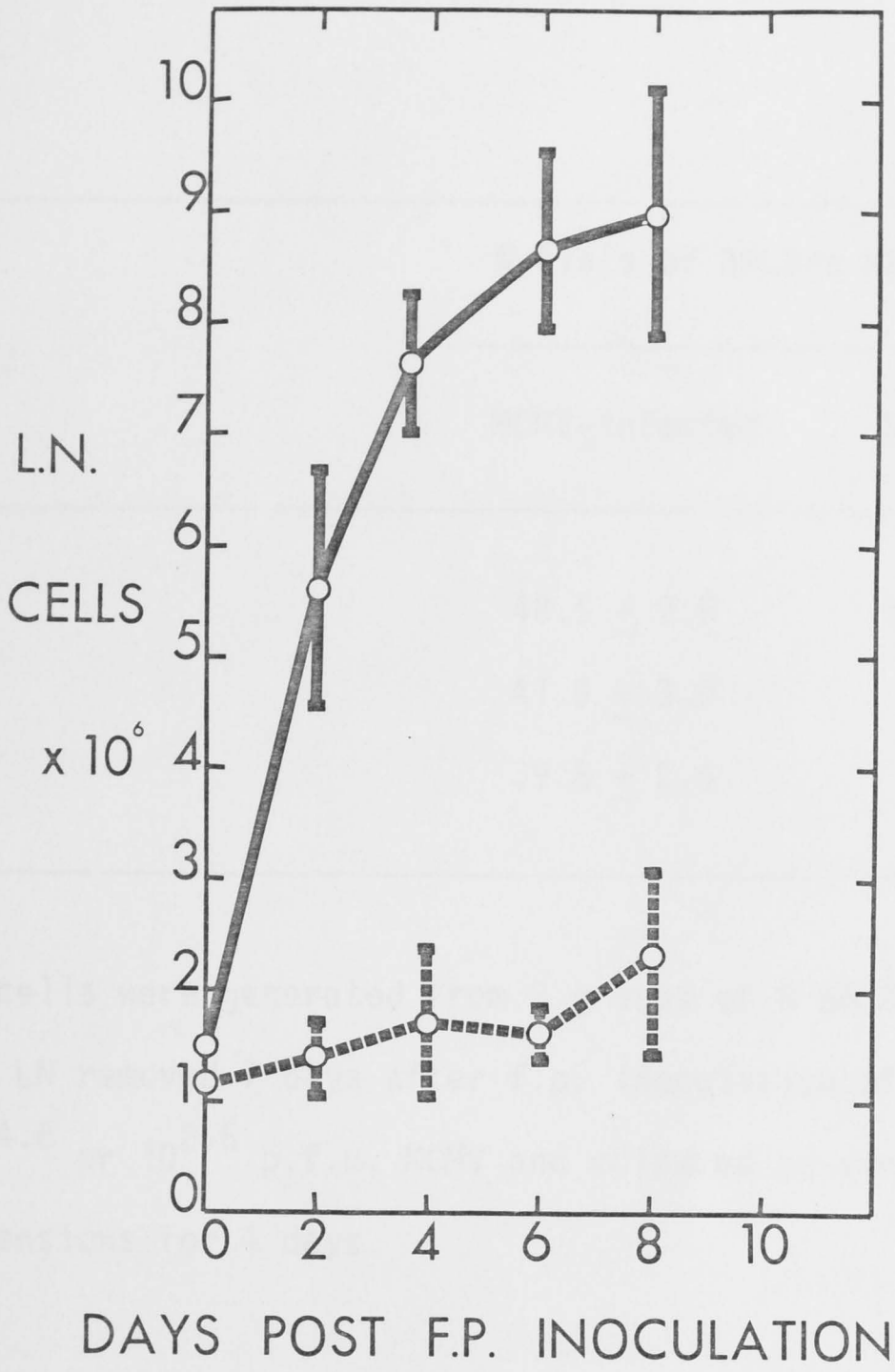

Fig. 5.

Cell numbers in the draining popliteal LN following hind f.p. inoculation with $10^{4.6}$ p.f.u. of MCMV (o——o) and normal salivary gland suspension (o----o). Each point represents the mean numbers of viable cells per popliteal LN ± standard deviation of the mean (in groups of 5).

TABLE 2

Effect of viral dose on cytotoxicity mediated by MCMV-immune cultured LN cells* against MCMV-infected target cells

Viral dose (p.f.u./f.p.)	% lysis of BALB/c MEF targets†	
	MCMV-infected	Uninfected
$10^{6.1}$	48.5 ± 2.6	14.5 ± 0.7
$10^{4.6}$	41.9 ± 3.0	5.5 ± 0.1
$10^{2.6}$	39.8 ± 2.4	6.4 ± 0.4

* Effector cells were generated from 3 groups of 6 BALB/c popliteal LN removed 7 days after f.p. inoculation of $10^{6.1}$, $10^{4.6}$ or $10^{2.6}$ p.f.u. MCMV and cultured as single-cell suspensions for 4 days.

† The % lysis values are the means ± standard errors of the mean of 3 replicates obtained by addition onto targets of a 1/36 aliquot of cultured cells from 6 BALB/c popliteal LN.

TABLE 3

Cytotoxic activity of LN cells from BALB/c mice 7 days after MCMV-infection assayed directly or after a further 4 days in culture

Effector cells	% lysis of BALB/c MEF targets[§]	
	MCMV-infected	Uninfected
*Direct LN assay	13.5 ± 1.9	9.8 ± 0.7
†Ig^+ cells removed	12.4 ± 1.0	14.6 ± 1.0
‡Positive control	72.8 ± 1.3	15.6 ± 0.5

* Popliteal LN cells were obtained from 3 BALB/c mice infected 7 days previously with $10^{4.6}$ p.f.u. of MCMV and assayed directly.

† LN cells obtained as in * above were treated as described in Materials and Methods to remove Ig^+ cells and assayed directly.

‡ The positive control effector cells were obtained as in * above, but were cultured for an additional 4 days.

§ % lysis values are means ± standard error of the mean of 3 replicates obtained by addition of a 1/12 aliquot of cells from 6 BALB/c popliteal LN.

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CHAPTER 3

The Cytotoxic Response to Murine Cytomegalovirus.
II. *In vitro* requirements for generation of cytotoxic T cells

INTRODUCTION

The generation of a cytotoxic T (Tc) cell response both *in vivo* and *in vitro* has been reported for many viruses (Zinkernagel & Doherty, 1979). Usually these T cell responses have been studied using either direct assay from primary spleen cell populations *in vivo* or restimulation *in vitro* of secondary spleen cell populations. The situation with the Beta-herpesvirus, murine cytomegalovirus (MCMV) has been more difficult in that reports of direct primary cytotoxicity are few and show low levels of cytotoxicity on MCMV-infected target cells (Quinnan *et al.*, 1979) or lack of reproducibility (Ho, 1980). Secondary stimulation *in vitro* with MCMV has been reported (Ho, 1980), but in our hands cytotoxicity against MCMV-infected targets over and above that expressed against uninfected targets has been low and unreliable.

However, now that we can consistently generate cytotoxicity against MCMV-infected targets above that observed for uninfected targets by a combination of *in vivo* and *in vitro* methods (Sinickas *et al.*, 1984, submitted) we have been able to characterise the effector population and the *in vitro* conditions needed to optimize specific anti-MCMV specificity.

METHODS

Mice. BALB/c, CBA/H, and WEHI-3 mice, 6-12 weeks of age, were obtained from the Animal Breeding Establishment of the John Curtin School of Medical Research.

Viruses. Standard methods were used for preparation of the Smith strain of MCMV (Sinickas et al., 1984, submitted) and influenza A/WSN (Yap & Ada, 1977).

Generation of antiviral effector cells. Anti-MCMV effector cells were generated as described previously (Sinickas et al., 1984, submitted) with variations given in details in Results. Anti-influenza A/WSN effector cells were generated by secondary cultures of primed spleen cells as described by Yap & Ada (1977).

Target cell culture. The DBA/2 mastocytoma cell line P-815-X2 (P815) and mouse embryo fibroblasts (MEF), obtained by trypsin dispersion, were grown at 37°C in a humidified atmosphere containing 10% CO_2, 7% O_2 and 83% N_2 (special gas) in Dulbecco's Modified Eagle's Medium (Cat. No. H16, GIBCO, Grand Island, N.Y., U.S.A.) supplemented with 5% foetal calf serum (FCS) (Flow Labs, Stanmore, N.S.W., Australia), 200 ug/ml Streptomycin, 200 U/ml Penicillin G and 150 ug/ml Neomycin sulphate (antibiotics). (The complete medium is referred to as DMEM.)

Cytotoxicity Assay. $10^{6.3}$ P815 cells in 0.5 ml of DMEM were infected with 0.2 ml of influenza A/WSN stock (allantoic fluid

Haemagglutinin titre 1:1024) for 60 mins at 37°C in special gas. Subsequently 600 uCi $Na_2[^{51}Cr]O_4$ (Amersham Int. Ltd., Amersham, U.K.) in 5 ml of DMEM was added and incubation continued for 2 hrs, after which time the cells were centrifuged, washed and resuspended at $10^{5.3}$ cells/ml. Aliquots of 100 ul were added to 96-well round-bottomed microtitre wells (Nunc, Roskilde, Denmark) followed by 100 ul of effector cells suspended in Eagle's Minimal Essential Medium (Cat. NO. F15, GIBCO, Grand Island, N.Y., U.S.A.) supplemented with 5% FCS and antibiotics (Assay medium). Cell-free supernatants were harvested after 10 hrs incubation as described for MEF. The MEF target cell protocol with MCMV, assay of gamma emissions, and calculations for cytotoxicity were unchanged from that previously described (Sinickas et al., 1984, submitted).

Antiserum treatments. Cultured or uncultured popliteal lymph node (LN) cells were collected and suspended at $10^{7.6}$ cells/ml in assay medium containing 100 ul of 1:40 dilution of Thy1.2 antiserum (Clone F7D5, Olac 1976 Ltd., Oxon., England) per 10^8 cells. After incubation at 4°C for 45 minutes, the cells were washed twice and resuspended at $10^{7.0}$ cells/ml in assay medium containing a 1:8 dilution of low-Tox-M rabbit complement (C') (Cedarlane Laboratories Ltd., Ontario, Canada). After 45 mins incubation at 37°C, the cells were washed twice with assay medium and either utilised as effector cells in cytotoxicity assays or cultured.

The Lyt2 antiserum 3.168 was generously provided by Dr. J. Allen (originally described by Sarmiento et al., 1980) and was used to treat LN cells at a cell concentration of $10^{7.2}$ cells/ml in assay medium with addition of 50 ul of 1:20 dilution of Lyt2

antiserum per $10^{7.3}$ cells. Washing procedures and C' treatment was as described above for anti-Thy1.2.

Gamma radiation. LN cells were suspended at $10^{7.3}$ cells/ml in assay medium and 10^{-4}M 2-mercaptoethanol (2ME) in siliconized test tubes and exposed to 400, 800, 1200 or 1600 rads from a ^{60}Co source.

Thymidine incorporation. On days 0, 1, 2, 3 and 4 of culture, 0.1 ml aliquots of LN cells were removed from culture vessels (Cat. No. 3512, Costar, Cambridge, Mass., U.S.A.) and placed into 96-well flat-bottomed microtitre trays (Nunc, Roskilde, Denmark). To each well, 50 ul of [methyl-^{3}H]-Thymidine (Amersham Int. Ltd., Amersham, U.K.) at 25 uCi/ml was added. After 5 hrs incubation at 37^{o}C in special gas, the cells were harvested onto glass fibre paper using a MASH II multiple sample harvester (Microbiological Associates, Bethesda, Md., U.S.A.). The dried samples of glass fibre paper were placed in 7 ml of scintillation fluid and beta emissions counted in a liquid scintillation counter (Tricarb, Packard Instrument Co., Downers Grove, Ill., U.S.A.). All samples were assayed in triplicate.

Mitomycin C treatment. LN cells were collected and suspended at $10^{7.0}$ cells/ml in Eagle's Minimal Essential Medium to which 20 ug of Mitomycin C (Sigma, St. Louis, Mo., U.S.A.) in phosphate-buffered saline (200 ug/ml) was added per $10^{7.0}$ cells. After 30 mins incubation at 37^{o}C, the cells were washed three times and cultured.

Concanavalin A activated spleen cell supernatant (CSS). This was prepared as previously described (Sinickas et al., 1984, submitted) using spleen cells from WEHI-3 mice.

RESULTS

An experimental program *in vitro* was undertaken to investigate the characteristics of the cytotoxic response against MCMV infection generated in cells from popliteal LN taken 7 days after hind footpad (f.p.) inoculation of MCMV and then cultured for 4 days as described in the preceding paper (Sinickas *et al.*, 1984, submitted).

Effector:Target (E:T) cell ratio

Six BALB/c popliteal LN were harvested at day 7 after bilateral f.p. inoculation of $10^{4.6}$ plaque-forming units (p.f.u.) MCMV. Single cell suspensions were prepared and cultured without additional virus. On day 4 of culture the LN cells were harvested and assayed for cytotoxicity on MCMV-infected and uninfected BALB/c MEF targets. We have arbitrarily chosen to use pooled cells from six LN as one cultured unit and hence the effector:target (E:T) ratios are expressed as the reciprocal of the fraction of this unit (as $\log_{10}$), placed onto the targets in the cytotoxicity assay. In this example there was significant lysis of MCMV-infected MEF at the 3 highest E:T ratios (Fig. 1) and lysis of uninfected MEF only at the highest E:T ratio. In other experiments, lysis of uninfected MEF in relation to lysis of infected MEF was sometimes higher and sometimes lower than the example shown in Fig. 1.

Effect of anti-Thy1.2 serum, viral specificity and H-2 restriction

BALB/c and CBA/H female mice were inoculated into both hind f.p. with $10^{2.6}$ p.f.u. of MCMV and units of 6 popliteal LN from mice of each strain were used after 7 days to prepare single-cell suspensions. The LN cells were cultured for 4 days, harvested and subjected to treatment with Thy1.2 antiserum and C', C' treatment alone, or left untreated prior to cytotoxicity assay. Anti-influenza A/WSN effector cells were generated by *in vitro* restimulation of $10^{8.0}$ primed spleen cells with $10^{7.0}$ spleen cells infected with influenza A/WSN (Yap & Ada, 1977). Spleen cells were harvested on day 5 of culture and used in cytotoxicity assays.

Target cells utilised were MCMV-infected or uninfected MEF from BALB/c(H-2^d) or CBA/H(H-2^k) mice, and influenza A/WSN-infected or uninfected P815(H-2^d) cells. MCMV-infected MEF were lysed by effector cells that were sensitive to treatment with Thy1.2 serum and C' (Table 1), were H-2-restricted (Table 2), and were virus-specific with respect to MCMV and influenza A/WSN (Table 3). These are the properties of antiviral Tc cells.

Effect of anti-Lyt2 serum upon cytotoxicity

Nine BALB/c female mice were inoculated into both hind f.p. with $10^{2.6}$ p.f.u. MCMV. Draining popliteal LN were removed on day 7 post inoculation when single-cell suspensions were prepared and cultured for 4 days. The cells were then harvested, pooled at $10^{7.6}$ cells/ml in assay medium, divided into 3 equal aliquots and

subjected to treatment with C' alone, Lyt2 antiserum and C', or left untreated.

The recovery of viable cells after anti-Lyt2 plus C' treatment and after C' alone was 75% and 80% respectively. Cytotoxicity was tested after all the aliquots were adjusted to equal volumes and 1/108 aliquot of the adjusted volume added to MCMV-infected or uninfected MEF. Lyt2 antiserum and C' treatment abrogated the cytotoxic activity compared to C' alone or no treatment (Table 4).

Effects of temperature and CSS

Draining popliteal LN from 12 BALB/c female mice were removed 7 days after bilateral hind f.p. inoculation with $10^{2.6}$ p.f.u. MCMV. The cells from 24 LN were pooled, adjusted to $10^{5.8}$ cells/ml, divided into 4 aliquots which were cultured for 4 days (a) at 34°C with CSS, (b) at 37°C with CSS, (c) at 34°C without CSS, (d) at 37°C without CSS, and then assayed for cytotoxic activity against MCMV-infected and uninfected BALB/c MEF.

34°C and 37°C were used in an attempt to duplicate popliteal LN temperature and visceral body temperature respectively. However, incubation temperature had no effect upon Tc cell generation, but the presence of CSS significantly increased cytotoxic activity against both infected and uninfected target cells (Table 5).

LN cell concentration in vitro

Popliteal LN from 12 BALB/c female mice inoculated into both hind f.p. with $10^{2.6}$ p.f.u. MCMV were obtained on day 7. A single-cell

suspension pool was prepared, divided into 4 aliquots and then cultured at concentrations of $10^{6.8}$, $10^{6.3}$, $10^{5.8}$, $10^{5.3}$ cells/ml. On day 4 the cells were harvested and assayed for cytotoxicity against MCMV-infected and uninfected BALB/c MEF. Cell concentrations of $10^{5.8}$-$10^{5.3}$ cells/ml were optimal for generation of MCMV-specific Tc cells (Table 6).

Effect of gamma irradiation

To determine whether proliferation *in vitro* of cells derived from MCMV-stimulated popliteal LN was required for Tc cell generation, LN cells were subjected to various doses of gamma irradiation prior to culture. Twenty BALB/c mice were inoculated with $10^{2.6}$ p.f.u. MCMV into both hind f.p. On day 7 the forty popliteal LN were removed, and a cell suspension was prepared at $10^{7.3}$ cells/ml in assay medium supplemented with 10^{-4} 2ME. This suspension was divided into 5 aliquots which were subjected to 0, 400, 800, 1200 or 1600 rads of gamma irradiation from a ^{60}Co source. One quarter of the cells of each aliquot was cultured at $10^{5.8}$ cells/ml for 4 days. On days 0, 1, 2 and 4 of culture, triplicate 0.1 ml samples were removed from each culture vessel and assayed for [^{3}H]-thymidine incorporation. The remaining three quarters of each aliquot were cultured for 4 days after which a 1/12 fraction was placed onto MCMV-infected and uninfected BALB/c MEF targets for a cytotoxicity assay.

[^{3}H]-thymidine incorporation increased over the 4 days in culture for the unirradiated cell population, whilst for irradiated cells proliferation was decreased, with an inverse relationship

between radiation dose and proliferative response (Fig. 2). Concomitant with the decreased proliferation, there was decreased cytotoxicity against MCMV-infected targets (Table 7). An experiment utilizing Mitomycin C instead of gamma irradiation revealed complete abrogation of cytotoxic activity on day 4 (data not shown).

Effect of Thy1.2 antiserum and C' treatment on anti-MCMV LN cells prior to culture

Twelve BALB/c female mice were inoculated with $10^{2.6}$ p.f.u. MCMV into both hind f.p. On day 7 the 24 popliteal LN were removed and a cell suspension was prepared at $10^{7.6}$ cells/ml in assay medium. This suspension was divided into 3 equal aliquots which were treated with Thy1.2 serum plus C', C' alone or assay medium only. (The recovery of viable popliteal LN cells after anti-Thy1.2 plus C' treatment and after C' alone was 55% and 80% respectively.) One quarter of the cells from each aliquot was cultured at $10^{5.8}$ cells/ml for 4 days. On days 0, 1, 2, 3 and 4 of culture replicates of six 0.1 ml samples were removed from each culture vessel and assayed for [^{3}H]-thymidine incorporation. The remaining 3 quarters of each aliquot were cultured for 4 days, after which a 1/36 fraction was placed onto MCMV-infected and uninfected BALB/c MEF targets for a cytotoxicity assay.

[^{3}H]-thymidine incorporation increased to a peak at 3 days for the LN cells that were untreated or subjected to C' alone (Fig. 3). Anti-Thy1.2 plus C' treatment decreased LN cell proliferation (Fig. 3). Cytotoxicity against MCMV-infected targets by LN cells treated with anti-Thy1.2 plus C' was markedly decreased compared

with cells treated with C' alone or untreated (Table 8).

Effect of Lyt2 antiserum and C' treatment on anti-MCMV LN cells prior to culture

Fifteen BALB/c female mice were inoculated with $10^{2.6}$ p.f.u. MCMV into both hind f.p. and on day 7 the 30 popliteal LN were removed and a cell suspension prepared in assay medium. This suspension was divided into 3 equal aliquots which were treated with anti-Lyt2 serum plus C', C' alone or assay medium only. The recovery of viable popliteal LN cells after anti-Lyt2 serum plus C' treatment or C' alone treated cells was 86% and 81% respectively.

One-fifth of the cells from each aliquot was cultured at $10^{5.8}$ cells/ml for 4 days and another fifth was cultured in two equal portions with or without 5 ug/ml Concanavalin A at $10^{6.0}$ cells/ml in assay medium plus 10^{-4}M 2ME without CSS for 4 days. On days 0, 1, 2, 3 and 4 of culture, replicates of six 0.1 ml samples were removed from each culture vessel and assayed for [^{3}H]-thymidine incorporation. The remaining three-fifths of each aliquot were cultured for 4 days in assay medium plus 2ME with CSS, after which a 1/36 fraction was placed onto MCMV-infected and uninfected BALB/c targets for a cytotoxicity assay.

In the absence of Concanavalin A, [^{3}H]-thymidine incorporation increased to a peak at 3 days, for the LN cells that were subjected to C' alone or untreated (Fig. 4). The cells treated with anti-Lyt2 plus C' showed a delayed onset of increased [^{3}H]-thymidine incorporation (day 3) and never achieved the same level of activity as the untreated control cells during the four-day culture.

Cytotoxicity (Table 9) was markedly reduced by the Lyt2 antiserum plus C' treatment.

The LN cells stimulated by Concanavalin A showed increased [^{3}H]-thymidine incorporation to a peak on day 3; the temporal profiles and the peak levels attained were similar, irrespective of antiserum treatments (data not shown).

DISCUSSION

In this investigation popliteal LN cells taken 7 days after bilateral hind f.p. inoculation of MCMV and then cultured for 4 days have been used to further characterise the cytotoxic cells thus generated which lyse MCMV-infected target cells, and to define the conditions *in vitro* which support this response. The cytotoxic cells were destroyed by both Thy1.2 antiserum or Lyt2 antiserum and complement, were specific for MCMV in comparison with influenza virus, and were apparently H-2-restricted in that they did not lyse allogeneic MCMV-infected target cells. These properties are characteristic of virus-specific Tc cells described previously (Blanden *et al.*, 1975; Ada *et al.*, 1981).

The conditions under which the popliteal LN cells were cultured for 4 days determined the magnitude of the anti-MCMV Tc cell response. The addition of CSS to the culture medium significantly increased cytotoxic activity against both MCMV-infected and uninfected self MEF target cells, suggesting firstly that inadequate interleukin production may be a limiting factor in the anti-MCMV response, and secondly that the observed autoreactivity may be T cell-mediated as in other antiviral responses studied previously (Blanden & Gardner, 1976). However, it may be significant that the optimum LN cell concentrations for anti-MCMV Tc cell generation were $10^{5.3}$ to $10^{5.8}$ per ml. This is 2- to 10-fold less than the $10^{6.3}$ per ml established as the optimum for other primary and secondary anti-viral responses (Blanden *et al.*, 1977; Gardner & Blanden, 1976) and for the generation of Tc cells in mixed lymphocyte reactions (Lafferty *et al.*, 1974). It seems possible that

this phenomenon may reflect a need to dilute an inhibiting influence on Tc cell development that could be present in MCMV- or HSV-infected lymphoid tissues *in vivo* (Pfizenmaier *et al.*, 1976), but thus far we have not identified such an inhibitory factor.

Both proliferation of the popliteal LN cell population, and the generation of anti-MCMV Tc cells were decreased by gamma irradiation, mitomycin C treatment or anti-Thy1.2 plus complement of the responding popliteal LN cells taken 7 days after MCMV inoculation and before 4 days of culture. These experiments, together with the need for CSS, indicate that clonal expansion of anti-MCMV Tc cells was required during the 4 days of culture in order for them to reach detectable levels. Therefore it seems unlikely that sufficient clonal expansion had occurred *in vivo* by day 7 after infection, with only differentiation of effector function being required *in vitro* (Pang & Blanden, 1976; Raulet & Bevan, 1982). Addition of purified interleukin-2 to the 4-day culture step could further clarify whether proliferation alone or with differentiation is required for effector Tc cell activity to develop fully in this system.

The idea that $Lyt1^+2^-$ helper T cell function required for full clonal expansion of $Lyt1^-2^+$ antiviral Tc cells (Ashman & Müllbacher, 1979) is lacking in MCMV-infected mice was consistent with results obtained by treating anti-MCMV LN cells harvested 7 days post-infection with anti-Lyt2 plus complement prior to 4 days of culture. Such treatment killed a numerically insignificant proportion of the LN cell population since > 80% viable cell recovery was obtained after both anti-Lyt2 plus C', and C' treatment alone. In contrast, anti-Thy1.2 plus complement gave a viable cell recovery of only 55%.

As expected from these data, anti-Lyt2 plus C' treated LN cell populations contained functional T cells as indicated by their proliferative response to Con A. However, there must have been very few $Lyt2^-$ anti-MCMV T cells (putative helper T cells) present or capable of proliferation under the culture conditions~~, since very little proliferation of this population took place~~ in the absence of Con A. Also as expected, anti-Lyt2 plus C' treatment of 7-day anti-MCMV LN cells markedly impaired the development *in vitro* of the anti-MCMV cytotoxic potential of these cells, thus suggesting that the majority of anti-MCMV Tc cells were $Lyt2^+$, similar to other anti-viral Tc cells (Ada *et al.*, 1981).

SUMMARY

A cytotoxic response to murine cytomegalovirus was obtained by the culture of lymph node cells from mice inoculated with MCMV into both hind footpads seven days previously. The cytotoxicity was mediated by Thy1.2$^+$, Lyt2$^+$, H-2 restricted effector cells and was virus-specific. Investigation of the *in vitro* conditions established that T cell proliferation was necessary for optimal generation of cytotoxicity, that proliferation was dependent upon Thy1.2$^+$, Lyt2$^+$ cell populations and that supernatants from Concanavalin A activated spleen cells enhanced the levels of cytotoxicity obtained.

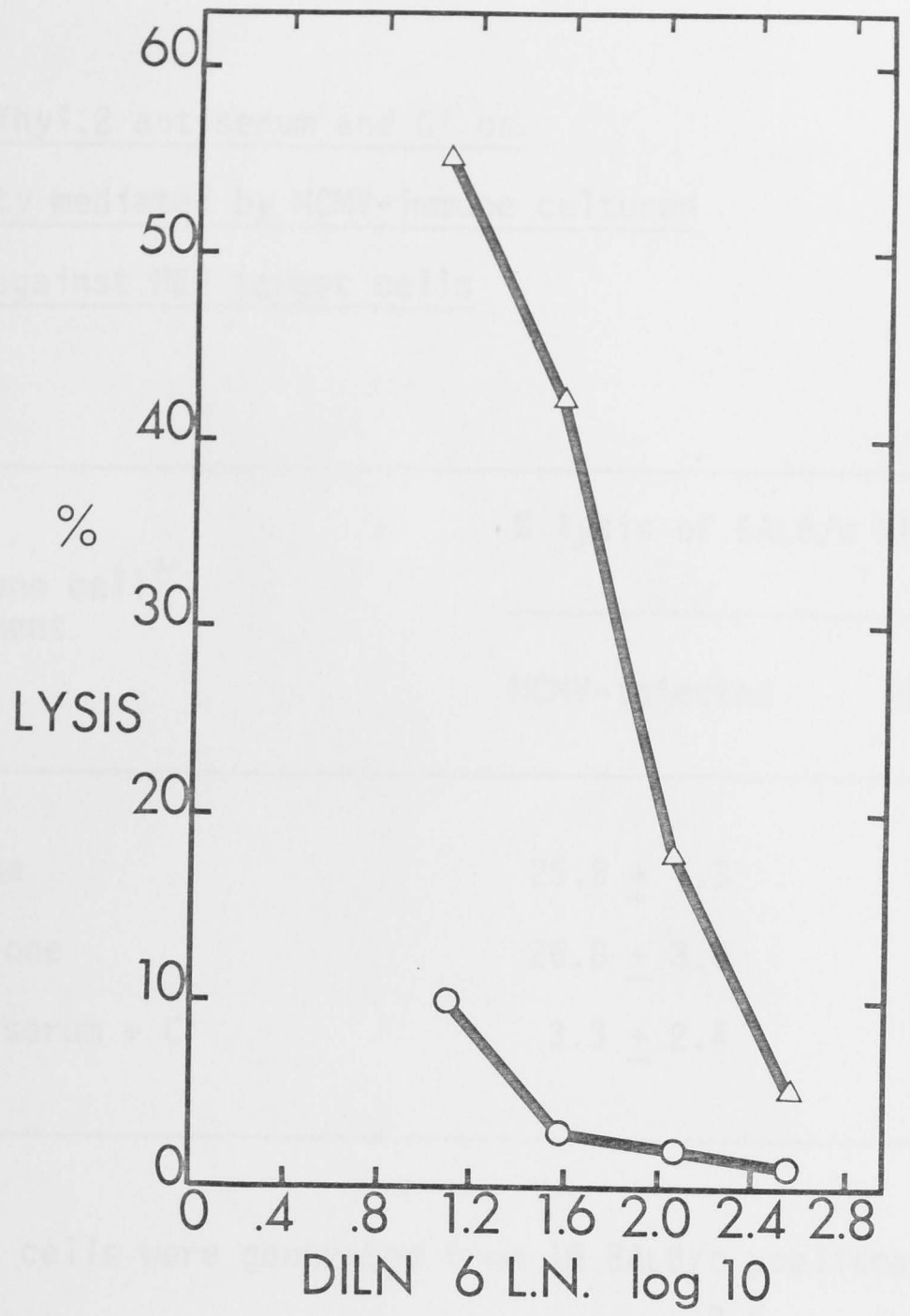

Fig. 1.

Cytotoxic activity of popliteal LN cells generated from 3 BALB/c mice after bilateral hind f.p. inoculation with $10^{4.6}$ p.f.u. MCMV. The LN cells were harvested on day 7 after inoculation and cultured for 4 days. Tripling dilutions of 1/12 aliquot of cultured LN cells were placed onto MCMV-infected (△) and uninfected (○) BALB/c MEF targets. E:T ratio is expressed as the $\log_{10}$ of the reciprocal of the fraction of the culture placed onto targets. In this experiment the highest E:T ratio = 90:1. The standard errors of the mean percent lysis were less than 4%. All cytotoxicity assays were performed in triplicate.

TABLE 1

Effect of Thy1.2 antiserum and C' on cytotoxicity mediated by MCMV-immune cultured LN cells* against MEF target cells

MCMV-immune cell† Treatment	% lysis of BALB/c MEF targets‡	
	MCMV-infected	Uninfected
None	25.9 ± 1.3	2.5 ± 0.6
C' alone	26.8 ± 3.6	5.7 ± 1.9
Thy1.2 antiserum + C'	3.3 ± 2.4	0.7 ± 0.9

* Effector cells were generated from 18 BALB/c popliteal LN removed 7 days after f.p. inoculation of $10^{2.6}$ p.f.u. MCMV and cultured as single-cell suspensions for 4 days. This cell pool was divided into 3 equal aliquots for different treatments prior to cytotoxicity assay.

† Conditions for antiserum treatment and assay are described in Materials and Methods.

‡ The % lysis values are the means ± standard errors of the mean of 3 replicates obtained by addition of a 1/12 aliquot of cultured cells from 6 BALB/c popliteal LN onto targets.

TABLE 2

H-2 restriction of anti-MCMV Tc cells* from BALB/c and CBA/H mice

Tc cell strain	% specific lysis of MCMV-infected MEF targets†	
	BALB/c	CBA/H
BALB/c	38.6	-12.2
CBA/H	1.8	32.1

* Effector cells were generated from 12 BALB/c and 12 CBA/H popliteal LN removed 7 days after f.p. inoculation of $10^{2.6}$ p.f.u. MCMV and cultured as single-cell suspensions for 4 days.

† % specific lysis = % lysis of MCMV-infected MEF - % lysis of uninfected MEF obtained by addition of a 1/12 aliquot of cultured cells from 6 BALB/c and 6 CBA/H popliteal LN onto targets. % lysis values are the means of 3 replicates. The standard errors of the mean were $<2.1\%$.

TABLE 3

Viral specificity of anti-MCMV BALB/c Tc cells

Tc cell type	% lysis of targets‡			
	MCMV-infected BALB/c MEF	Uninfected BALB/c MEF	Influenza A/WSN infected P815	Uninfected P815
Anti-MCMV*	33.1 ± 0.4	2.1 ± 0.4	9.0 ± 0.4	2.3 ± 0.3
Anti-influenza† A/WSN	3.2 ± 0.5	4.1 ± 0.8	89.7 ± 0.4	14.3 ± 6.4

* Effector cells were generated from 12 BALB/c popliteal LN removed 7 days after f.p. inoculation of $10^{2.6}$ p.f.u. MCMV and cultured as single-cell suspensions for 4 days.

† Anti-influenza A/WSN effector cells were generated using secondary cultures of primed spleen cells (Yap & Ada, 1977).

‡ The % lysis values are the means ± standard errors of the mean of 3 replicates. In the case of anti-MCMV LN cells, a 1/108 aliquot of cultured cells from 6 BALB/c popliteal LN was added to targets (an approximate E:T ratio of 15:1). In the case of anti-influenza spleen cells an E:T ratio of 15:1 was used.

TABLE 4

Effect of Lyt2 antiserum and C' on cytotoxicity mediated by MCMV-immune cultured LN cells* against MEF target cells

MCMV-immune† cell treatment	% lysis of BALB/c MEF targets‡	
	MCMV-infected MEF	Uninfected MEF
None	55.6 ± 1.6	8.5 ± 0.6
C' alone	72.7 ± 1.1	4.8 ± 0.2
Lyt2 antiserum + C'	2.6 ± 0.1	2.1 ± 0.2

* Effector cells were generated from 18 BALB/c popliteal LN removed 7 days after f.p. inoculation of $10^{2.6}$ p.f.u. MCMV and cultured as single-cell suspensions for 4 days. This cell pool was divided into 3 equal aliquots for various treatments prior to cytotoxicity assay.

† Conditions for antiserum treatment are described in Materials and Methods.

‡ The % lysis values are the means ± standard errors of the mean of 3 replicates obtained by addition of a 1/108 aliquot of cultured cells from 6 popliteal LN onto targets.

TABLE 5

The effect of temperature and CSS upon anti-MCMV Tc cell generation in vitro*

Culture Conditions	% lysis of BALB/c MEF targets†	
	MCMV-infected	Uninfected
CSS+, 34°C	52.6 ± 1.4	18.4 ± 1.0
CSS+, 37°C	55.1 ± 0.7	15.5 ± 0.4
CSS-, 34°C	28.0 ± 1.0	5.3 ± 0.7
CSS-, 37°C	27.1 ± 2.1	5.1 ± 0.5

* Effector cells were obtained from 24 BALB/c popliteal LN removed 7 days after f.p. inoculation of $10^{2.6}$ p.f.u. MCMV and cultured as single-cell suspensions for 4 days with or without CSS at different temperatures.

† The % lysis values are the means ± standard errors of the mean of 3 replicates obtained by the addition of a 1/12 aliquot of cultured cells from 6 BALB/c popliteal LN onto targets.

TABLE 6

The effect of cell concentration upon anti-MCMV Tc cell generation in vitro*

Cells/ml	% lysis of BALB/c MEF targets†	
	MCMV-infected	Uninfected
$10^{6.8}$	39.7 ± 2.4	4.1 ± 0.3
$10^{6.3}$	51.6 ± 1.9	11.9 ± 1.0
$10^{5.8}$	69.1 ± 2.8	15.2 ± 0.1
$10^{5.3}$	68.9 ± 1.7	16.6 ± 0.6

* The effector cells were derived from 24 BALB/c popliteal LN removed 7 days after f.p. inoculation of $10^{2.6}$ p.f.u. MCMV and cultured at different cell concentrations, as single-cell suspensions for 4 days.

† The % lysis values are the means ± standard errors of the mean of 3 replicates obtained by the addition of a 1/36 aliquot of cultured cells from 6 BALB/c popliteal LN onto targets.

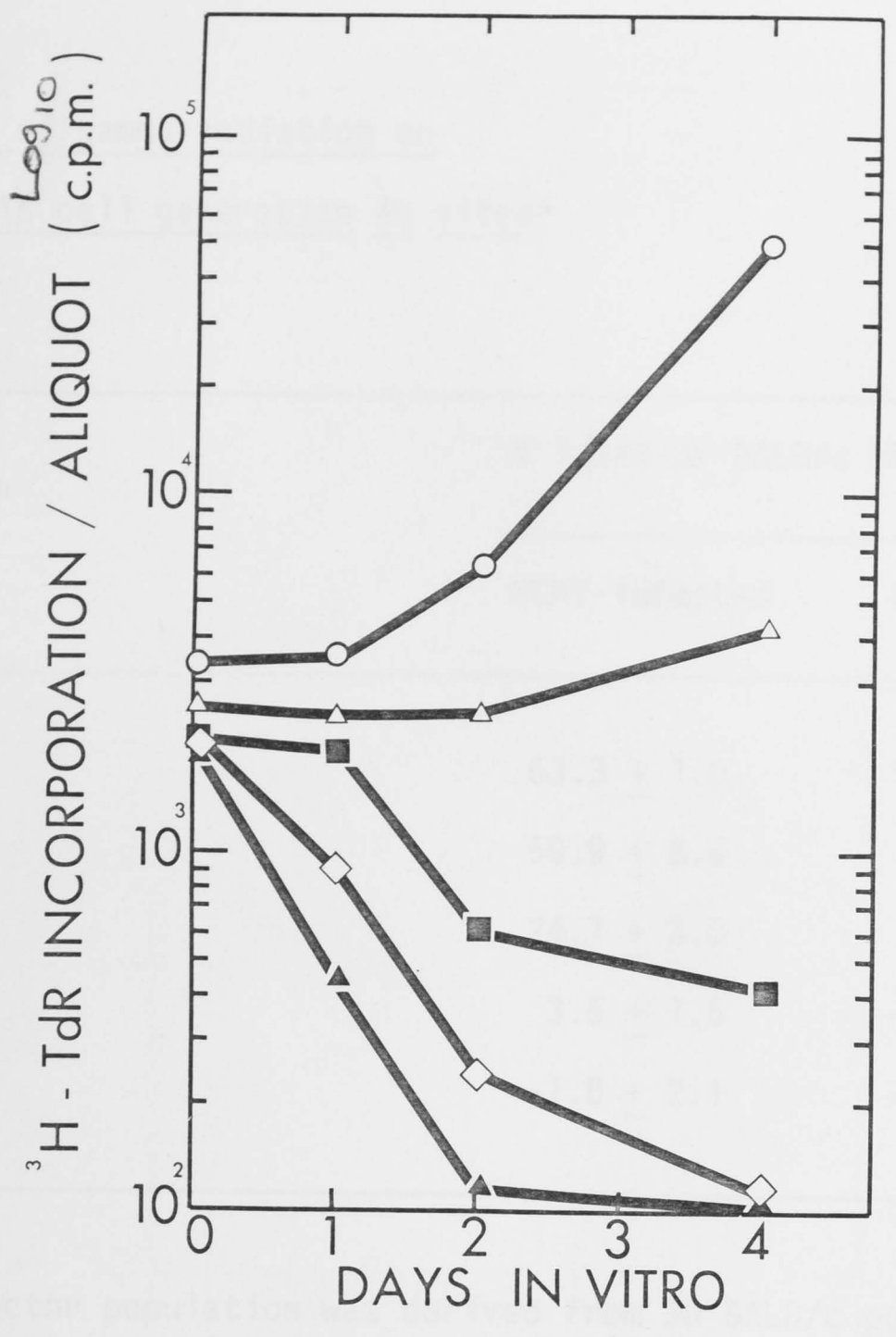

Fig. 2.

Effect of gamma radiation on proliferation *in vitro* of popliteal LN cells obtained on day 7 post f.p. inoculation of $10^{2.6}$ p.f.u. MCMV and treated with various doses of gamma irradiation prior to culture. 0.1 ml aliquots of cultured LN cells were assayed for [^{3}H]-thymidine incorporation on days 0, 1, 2 and 4. Data points are means of triplicate counts per minute (c.p.m.) and the standard errors are smaller than the symbols in the figure. Background [^{3}H]-thymidine incorporation was 320 c.p.m. Gamma irradiation doses 0, 400, 800, 1200 and 1600 rads are represented by ○, △, ■, ◇, and ▲, respectively.

TABLE 7

The effect of gamma radiation on anti-MCMV Tc cell generation in vitro*

Radiation dose (rad)	% lysis of BALB/c MEF targets†	
	MCMV-infected	Uninfected
0	63.3 ± 1.0	18.6 ± 2.1
400	50.9 ± 8.6	9.9 ± 1.1
800	24.1 ± 3.0	1.4 ± 0.5
1200	3.5 ± 1.6	-1.5 ± 0.7
1600	-1.8 ± 2.1	-2.2 ± 1.0

* The effector population was derived from 30 BALB/c popliteal LN removed 7 days after f.p. inoculation of $10^{2.6}$ p.f.u. MCMV. These LN cells were irradiated prior to culture for 4 days.

† % lysis values are the means ± standard errors of the mean of 3 replicates obtained by addition of a 1/12 aliquot of cultured cells from 6 BALB/c popliteal LN onto targets.

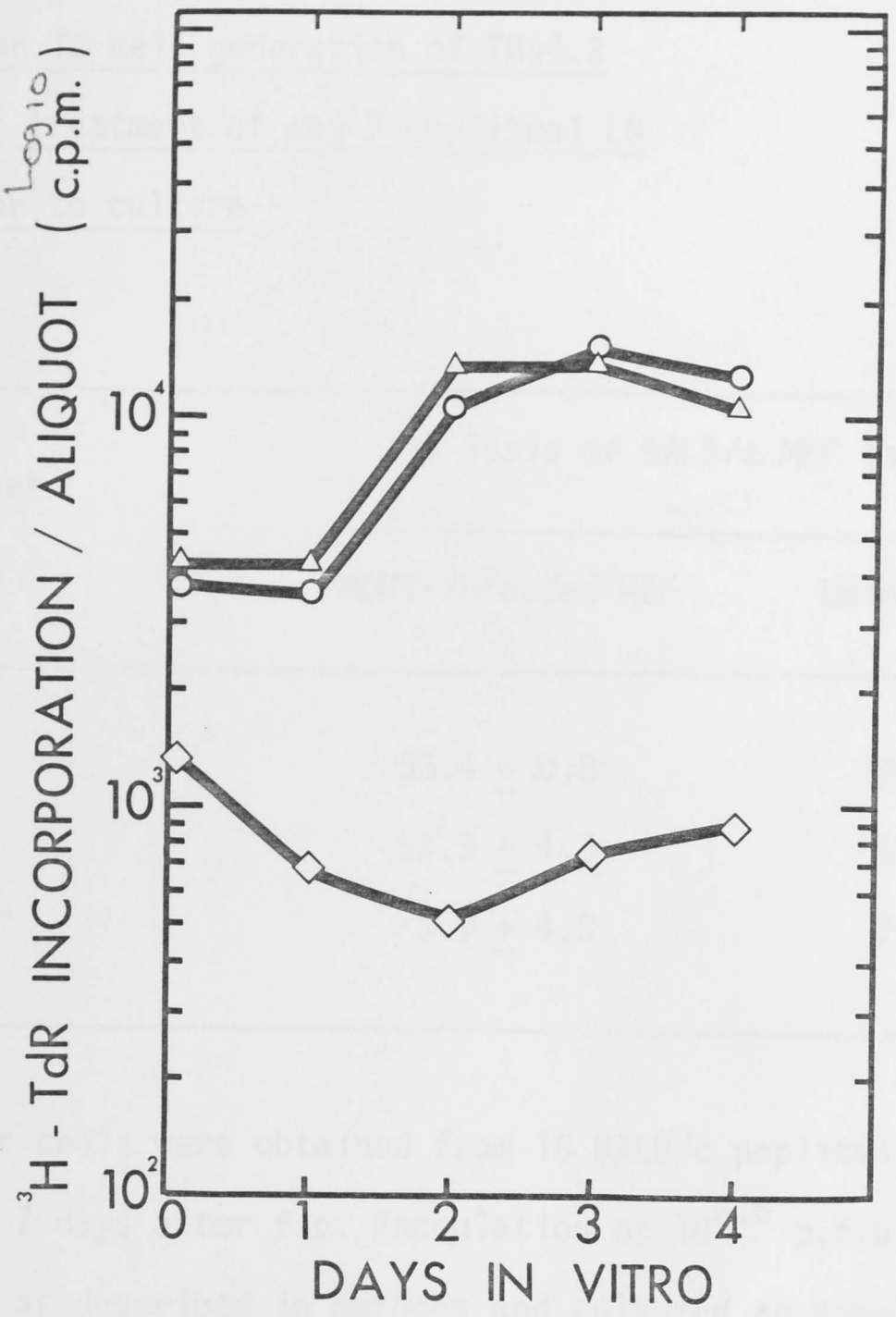

Fig. 3.

Effect of Thyl.2 antiserum + C' treatment on proliferation *in vitro* of anti-MCMV popliteal LN cells. Popliteal LN cells were removed on day 7 post f.p. inoculation of $10^{2.6}$ p.f.u. MCMV and treated with Thyl.2 antiserum + C' (◇), C' alone (△) or untreated (○). 0.1 ml aliquots of cultured LN cells were assayed for [^{3}H]-thymidine incorporation on days 0, 1, 2, 3 and 4. Data points are the mean c.p.m. ± standard deviations of the mean for six replicates. The error bars are smaller than the symbols in the figure. Background [^{3}H]-thymidine incorporation was 90 c.p.m.

TABLE 8

Effect upon Tc cell generation of Thy1.2 serum + C' treatment of day 7 popliteal LN cells prior to culture

MCMV-immune* cell treatment	% lysis of BALB/c MEF targets†	
	MCMV-infected MEF	Uninfected MEF
Nil	58.4 ± 0.8	10.0 ± 0.1
C' alone	55.3 ± 4.8	4.9 ± 0.5
Thy1.2 + C'	3.9 ± 4.8	7.1 ± 0.6

* Effector cells were obtained from 18 BALB/c popliteal LN removed 7 days after f.p. inoculation of $10^{2.6}$ p.f.u. MCMV, treated as described in Methods and cultured as single-cell suspensions for 4 days.

† % lysis values are the means ± standard errors of the mean of 3 replicates obtained by the addition of a 1/36 aliquot of cultured cells from 6 BALB/c popliteal LN onto targets.

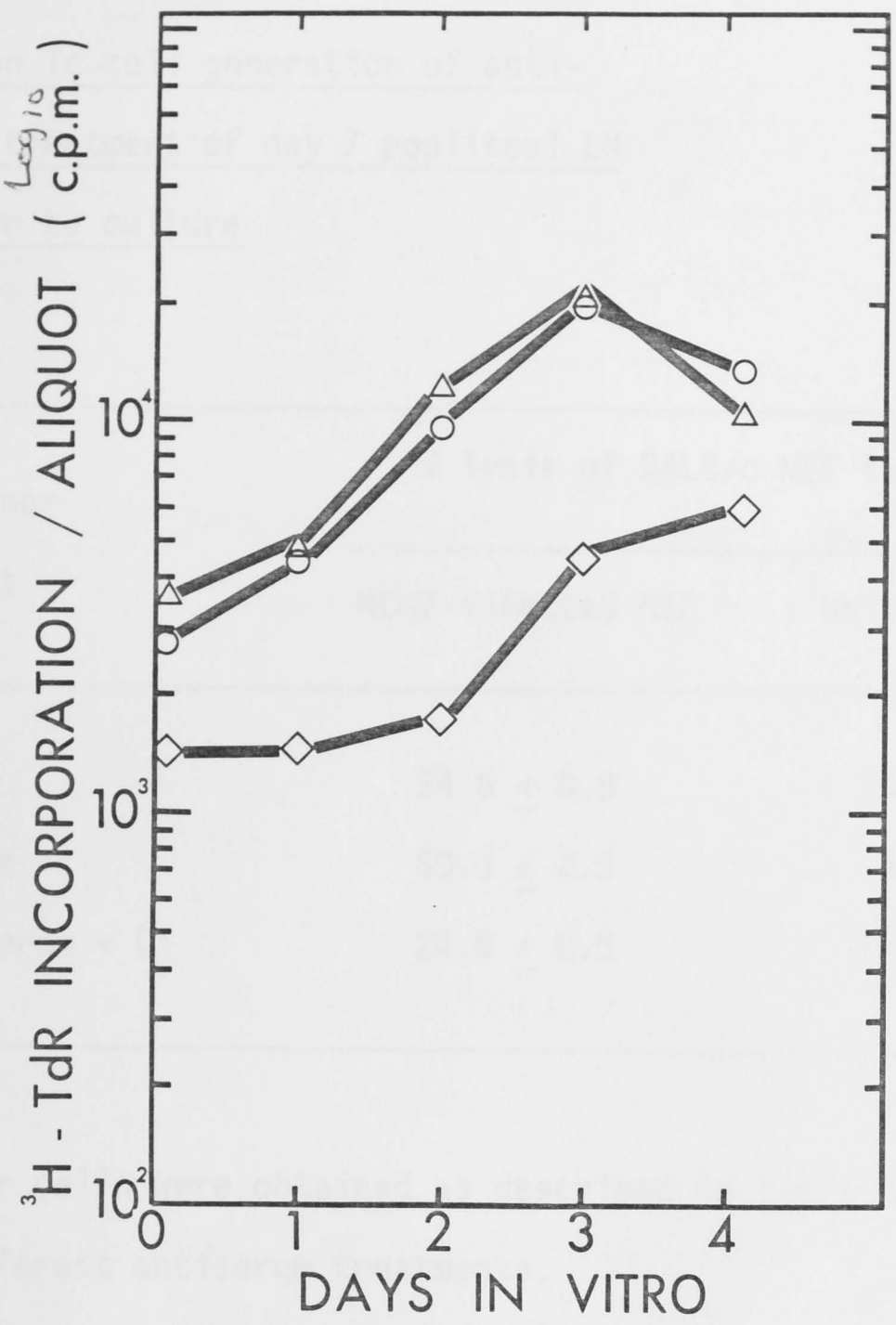

Fig. 4.

Effect of Lyt2 antiserum + C' treatment on proliferation *in vitro* of anti-MCMV popliteal LN cells. Popliteal LN cells were removed on day 7 post f.p. inoculation of $10^{2.6}$ p.f.u. MCMV and treated with Lyt2 antiserum + C' (◇), C' alone (△) or untreated (○). 0.1 ml aliquots of cultured LN cells were assayed for [^{3}H]-thymidine incorporation on days 0, 1, 2, 3 and 4. Data points are the mean c.p.m. ± standard deviations of the mean for six replicates. The error bars are smaller than the symbols in the figure. Background [^{3}H]-thymidine incorporation was <370 c.p.m.

TABLE 9

Effect upon Tc cell generation of anti-Lyt2 + C' treatment of day 7 popliteal LN cells prior to culture

MCMV-immune* cell treatment	% lysis of BALB/c MEF targets†	
	MCMV-infected MEF	Uninfected MEF
Nil	54.6 ± 0.9	14.2 ± 0.3
C' alone	50.3 ± 2.3	9.2 ± 0.2
Lyt2 antiserum + C'	24.6 ± 0.5	10.9 ± 0.4

* Effector cells were obtained as described in Table 8, except for different antiserum treatments.

† % lysis values were calculated as described in Table 8.

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CHAPTER 4

The Cytotoxic Response to Murine Cytomegalovirus.

III. Lymphokine release and cytotoxicity are dependent upon phenotypically similar immune cell populations

INTRODUCTION

Murine cytomegalovirus (MCMV) is a member of a sub-family of herpesviruses termed Betaherpesvirinae and as such is a useful model for the study of viral infections that usually result in non-fatal and latent illnesses. As part of the study of the immunobiology of MCMV infection we have investigated the cytotoxic T cell response to this virus, a response which may contribute to the clearance of infection (1). We have now utilised MCMV-immune lymph node (LN) cell populations to investigate the nature of T lymphocytes that release lymphokine after stimulation with MCMV. One particular lymphokine, Interleukin-3 (IL-3), a glycoprotein belonging to a family of glycoproteins known as colony-stimulating factors (CSF) that are essential for survival, growth and differentiation of haemopoietic progenitor cells *in vitro* (2), has been used in this study. We present evidence that IL-3 release from the MCMV-immune LN cell population was dependent upon Thy1.2$^+$, Lyt2$^+$ cells, that were triggered by Class I H-2-restricted and virus-specific antigen recognition: cytotoxic activity against MCMV-infected target cells was mediated by a phenotypically similar cell population.

MATERIALS AND METHODS

Mice. BALB/c, CBA/H, WEHI-3, B10.T(6R) and B10.AQR mice were obtained from the Animal Breeding Establishment of the John Curtin School of Medical Research and used between 6-12 weeks of age.

Viruses. The Smith strain of MCMV was obtained from Dr. G. Shellam, University of Western Australia. Salivary gland virus stock was prepared by intraperitoneal (i.p.) inoculation of $2x10^4$ plaque-forming units (p.f.u.)/0.2 ml into 4-week-old BALB/c female mice. At day 17 post inoculation, salivary glands were harvested and a 50% wt/vol homogenate prepared in Dulbecco's Modified Eagle's Medium (Cat. No. H16, GIBCO, Grand Island, N.Y., U.S.A.), supplemented with 5% foetal calf serum (FCS) (Flow Labs, Stanmore, N.S.W., Australia), 200 ug/ml Streptomycin, 200 U/ml Penicillin G and 125 ug/ml Neomycin sulphate (antibiotics). (The complete medium is referred to hereafter as DMEM.) Aliquots were stored at -70^oC. For dilution of virus stock 0.2M borate-buffered gelatin saline (pH 7.2-7.4) was used. Influenza A/WSN was prepared by standard methods (3).

Generation of Antiviral Immune Cells. Anti-MCMV immune LN cells were generated as described (4). Briefly, mice were inoculated into hind footpads (f.p.) with 40 ul of MCMV ($4x10^2$ p.f.u.). Seven days after inoculation, the draining popliteal LN cells were harvested and cultured at

$6.3x10^5$ cells/ml in 5 ml Costar wells (Cat. No. 3512, Costar, Cambridge, Mass., U.S.A.) containing Eagle's Minimal Essential Medium (EMEM) (Cat. No. F-15, GIBCO, Grand Island, N.Y., U.S.A.) supplemented with 10% FCS, antibiotics, 10^{-4}M 2-merceptoethanol (2ME) (Sigma Chemical Co., St. Louis, Mo., U.S.A.) + 3% vol/vol supernatant from Concanavalin-A-stimulated spleen cells (CSS). After 4 days incubation at 37°C in 83% N_2, 7% O_2 and 10% CO_2 (special gas) (Commonwealth Industrial Gases, Alexandria, N.S.W., Australia) effector cells were harvested. Anti-influenza A/WSN effector cells were generated by secondary cultures of primed spleen cells (3). After 5 days incubation the cells were harvested and debris removed by centrifugation through an Isopaque-Ficoll gradient.

Cell culture. Mouse embryo fibroblast (MEF) cultures were prepared from 16-18 day embryos by trypsin dispersion and growth in DMEM. 75 cm^2 tissue culture flasks (Nunc, Roskilde, Denmark) were seeded at $2x10^6$ cells/flask and incubated at 37°C for 4 days in special gas, after which time the cells were either subcultured immediately or kept at room temperature for up to 2 weeks prior to subculture. Conditions of subculture were the same as described for primary cultures, except that these secondary MEF cells were always used after 4 days at 37°C as the source for tertiary MEF target or stimulator cells.

The DBA/2 mastocytoma cell line P-815-X2 (P815) was grown in DMEM at 37°C in a humidified atmosphere containing 5% CO_2 in air.

The IL-3-dependent cell line, FDC-P1 (6) was grown in RPMI-1640 (Cat. No. 10-601-20, Flow Labs, Maclean, Virginia, U.S.A.)

supplemented with 5% heat-inactivated FCS (HI-FCS) and 10% WEHI-3B cell line conditioned medium (kindly provided by Dr. A.J. Hapel).

Preparation of Stimulator cells for Lymphokine production.

Secondary MEF were subcultured into 30 cm^2 plastic dishes (Cat. No. P.D.S. 6515H, Kayline Plastics, Thebarton, S.A., Australia) at 10^6 cells/10 ml DMEM, supplemented with 3% vol/vol CSS/dish. The cells were incubated for 2 days at 37°C in special gas, after which time the cell monolayers were washed twice with DMEM and adsorbed with 1 ml of 1:8 dilution of MCMV stock ($4x10^7$ p.f.u./ml) per dish. The cell monolayers were centrifuged at 800g for 20 mins at room temperature and then incubated at 37°C in special gas for 40 mins. Subsequently 10 ml of DMEM was added and incubation continued for 16 hrs. Uninfected control MEF were subjected to the same protocol, except no virus was added to the monolayer. At 16 hrs the cell monolayers were washed, trypsinized and resuspended in DMEM at 10^6 cells/ml, and 10 ml aliquots were then added to 65 cm^2 plastic petri dishes (Cat. No. 992550, Medical Plastics, Edwardstown, S.A., Australia) and subjected to ultraviolet (UV) irradiation from an 80 watt germicidal lamp for 4 mins at an intensity of 960 uW/cm^2 in the 230/270 nm range. These cells were then washed twice and resuspended at an appropriate concentration in EMEM supplemented with 10^{-4}M 2ME. Aliquots of 0.1 ml were dispensed into 96-well flat-bottomed trays (Nunc, Roskilde, Denmark). Serial twofold dilutions were prepared prior to addition of antiviral effector cells.

For P815 cells, 10^7 cells/500 ul DMEM were adsorbed with 200 ul influenza A/WSN stock (Haemagglutinin titre 1:1024) for

sixty minutes, after which 5 ml DMEM was added and incubation continued for another 6 hrs. Subsequent treatment for the influenza A/WSN-infected and uninfected P815 was similar to that described for MEF.

Conditions for stimulation of Lymphokine production. Antiviral effector cells were harvested, washed twice and resuspended in EMEM supplemented with 10^{-4}M 2ME at $5x10^6$ cells/ml. 100 ul aliquots were dispensed into 96-well flat-bottomed plates containing 100 ul aliquots of infected or uninfected stimulator cells. These cell mixtures were incubated at 37^{o}C in special gas mixture for 6 hrs, after which time cell-free supernatants were removed and stored at -20^{o}C until assayed for IL-3.

IL-3 Assay. IL-3 activity was assayed by measuring the ability of lymphokine preparations to maintain *in vitro* proliferation of the IL-3-dependent FDC-P1 cell line. After a period of 16-24 hrs without IL-3, FDC-P1 cells cease dividing. Serial twofold dilutions of the test supernatants were prepared in 96-well flat-bottomed plates (Linbro Cat. No. 76-203-05, Flow Labs, Maclean, Virginia, U.S.A.) with 50 ul RPMI-1640 plus 5% HI-FCS as diluent. Subsequently 50 ul of a FDC-P1 cell suspension ($4x10^5$) was added to each culture well. Control preparations consisted of 48 replicate cultures containing 50 ul of the FDC-P1 cell suspension and 50 ul of RPMI supplemented with 5% HI-FCS. The cultures were incubated at 37^{o}C for 16-20 hrs in 5% CO_2, after which time 50 ul of [methyl-^{3}H]-thymidine (Amersham Int. Ltd., Amersham, U.K.) (25 uCi/ml) were added and incubation continued for a further 5 hrs.

[Methyl-^{3}H]-thymidine incorporation was then determined by harvesting the cells onto glass fibre paper using a MASH II multiple sample harvester (Microbiological Associates, Bethesda, Md., U.S.A.). The dried samples of glass fibre paper were placed into 7 ml of scintillation fluid and beta-emission counted in a liquid scintillation counter (Tricarb, Packard Instrument Co., Downers Grove, Ill., U.S.A.). All samples were assayed in duplicate. The titration endpoint was set at three standard deviations (S.D.) above the mean control level of [methyl-^{3}H]-thymidine uptake, according to a previously described method (6).

Cytotoxicity assays. Previously described protocols for cytotoxicity assays using anti-influenza A/WSN (3) and anti-MCMV effector cells (4) were used. Briefly, 96-well flat-bottomed microtitre trays were seeded with tertiary MEF at 10^{4} cells/0.2 ml DMEM/well, 3 days prior to assay. After 2 days in culture, the cells were adsorbed with MCMV stock as described for stimulator cell preparation, except adsorption volume was 25 ul. Sixty minutes later, 150 ul of DMEM supplemented with 6 uCi of ^{51}Cr (Amersham Int. Ltd., Amersham, U.K.) was added and incubation continued at 37^{o}C in special gas for 16 hrs. Uninfected control MEF were treated in a similar manner except for no viral adsorption. The target cells were then washed twice, followed by addition of 100 ul of fresh DMEM and 100 ul of antiviral effector T cell suspension.

Triplicate cultures were set up for each dilution of antiviral effector cells and incubated for 10 hrs at 37^{o}C.

^{51}Cr release from the targets was measured and lysis calculated using the following formula:

$$\% \text{ lysis of infected or uninfected cells} = \frac{^{51}Cr \text{ counts in presence of effector cells} - ^{51}Cr \text{ counts released in medium}}{^{51}Cr \text{ counts from water-lysed targets} - ^{51}Cr \text{ counts released in medium}}$$

Antiserum treatments. Cultured MCMV-immune LN cells were harvested, centrifuged and resuspended in EMEM plus 5% FCS at $4x10^7$ cells/ml containing 300 ul of 1:40 dilution of Thy1.2 antiserum (Clone F7D5, Olac Ltd., Oxon., U.K.) per 10^8 cells. After incubation at 4^oC for 45 mins, the cells were washed twice and resuspended at 10^7/ml in EMEM plus 5% FCS containing a 1:8 dilution of low-Tox-M rabbit complement (C') (Cedarlane Laboratories Ltd., Ontario, Canada). After 45 mins incubation at 37^oC, the cells were harvested, washed twice and resuspended in EMEM and 10^{-4}M 2ME at $5x10^7$ cells/ml in preparation for stimulation of lymphokine production. Seven ul of neat Lyt2 antiserum 3.168 (8) (generously donated by Dr. J. Allen) was added to 0.95 ml cultured MCMV-immune LN cells suspended in EMEM plus 5% FCS at $1.4x10^6$ cells/ml. Treatment of cells subsequent to this step was as described for Thy1.2 antiserum and C' treatment, except that a 1:6 dilution of C' was used during the lytic phase.

Concanavalin-A-stimulated spleen cell supernatant (CSS). This was prepared as previously described (4) using spleen cells from WEHI-3 mice.

RESULTS

Effect of different numbers of MCMV-infected or uninfected MEF upon lymphokine release from MCMV-immune LN cells

Preliminary experiments investigated the optimal conditions for stimulation of IL-3 production and release from MCMV-immune LN cells obtained from various mouse strains. Three mice of each strain - BALB/c, B10.AQR and B10.T(6R) - were inoculated into both hind f.p. with $4x10^2$ p.f.u. MCMV. The popliteal LN cells were obtained on day 7 and cultured for 4 days, after which time they were harvested and resuspended at $5x10^6$ cells/ml in EMEM supplemented with 10^{-4}M 2ME. MCMV-infected or uninfected MEF were suspended at $2x10^7$ cells/ml and diluted in twofold steps down to $8x10^3$ cells/ml. Subsequently 100 ul MCMV-immune LN cells were mixed with 100 ul of MCMV-infected or uninfected MEF from the same mouse strain and incubated at 37^oC in special gas for 6 hrs.

The amount of IL-3 released from a fixed number of B10.AQR MCMV-immune LN cells ($5x10^5$) was directly proportional to the number of syngeneic MCMV-infected MEF added up to a maximum level between 1.2-$5x10^5$ MCMV-infected MEF (Fig.1). The results for the two other mouse strains tested were similar to the data obtained for B10.AQR. All subsequent lymphokine release experiments utilized $2x10^5$ MCMV-infected and uninfected MEF stimulators with $5x10^5$ MCMV-immune LN cells in a total volume of 200 ul.

Kinetics of IL-3 release after stimulation of MCMV-immune LN cells with MCMV-infected or Uninfected MEF

The temporal relationship between antigenic stimulation and IL-3 release from MCMV-immune LN cells was examined, so that an appropriate time point for harvest of reaction supernatants could be chosen. $5x10^5$ BALB/c MCMV-immune LN cells were stimulated with $2x10^5$ UV-irradiated MCMV-infected or uninfected BALB/c MEF in a total volume of 200 ul for 0, 1, 2, 4, 6, 8, 14 or 23 hrs, after which time cell-free supernatants were removed and assayed for IL-3 levels.

The results (Fig. 2) showed a maximum rate of increase in IL-3 titre between one and eight hrs after stimulation began. There was a further, slower increase in IL-3 titre until 14 hrs. With uninfected BALB/c MEF stimulators there was a maximum rate of increase of IL-3 titre from one to four hrs and a further, slower increase until 14 hrs, although the IL-3 titre reached was much lower than with infected MEF.

For logistic reasons all reaction supernatants were harvested 6 hrs after cell mixing in subsequent experiments.

Effect of Thy1.2 and Lyt2 antiserum treatment of MCMV-immune LN cells upon their ability to release IL-3

A batch of BALB/c MCMV-immune LN cells were harvested, divided into 4 equal portions and treated with C' alone, Thy1.2 antiserum and C', Lyt2 antiserum and C', or left untreated. Subsequently, the four populations of MCMV-immune LN cells were adjusted to the

same viable cell concentration ($5x10^5$ cells/100 ul). For lymphokine stimulation $5x10^5$ MCMV-immune LN cells were mixed with $2x10^5$ MCMV-infected or uninfected BALB/c MEF in a total volume of 200 ul.

The results (Table 1) revealed that IL-3 release dependent upon recognition of MCMV-infected MEF involved Thy1.2, $Lyt2^+$ immune LN cells, a phenotype similar to that of cytotoxic T lymphocytes. Thy1.2 antiserum plus complement treatment also resulted in a decrease in the IL-3 titre released upon stimulation with uninfected MEF or from MCMV-immune LN cells cultured alone, but Lyt2 antiserum and complement did not. These data suggest that $Lyt2^-$, $Thy1.2^+$ MCMV-immune T cells also exist and release IL-3, but that such T cells are not stimulated by MCMV-infected MEF.

H-2 restriction of IL-3 stimulation

MCMV-immune LN cells were obtained from the B10.T(6R) and CBA/H mouse strains. $5x10^5$ MCMV-immune LN cells from each strain were stimulated with $2x10^5$ MCMV-infected or uninfected MEF also derived from B10.T(6R) or CBA/H mice. The results (Table 2) showed that when the MCMV-immune LN cells and MEF stimulator cells were syngeneic, infected MEF stimulated considerably more IL-3 release than uninfected MEF. In contrast, when MCMV-immune LN cells and stimulator MEF were allogeneic, infected and uninfected MEF caused similar IL-3 release. These results are consistent with the interpretation that IL-3 release can follow H-2 restricted recognition of MCMV antigens by MCMV-immune T cells.

Viral specificity of MCMV-immune LN cells

BALB/c MCMV-immune LN cells and BALB/c influenza A/WSN-immune spleen cells were obtained and $5x10^5$ of each category of immune cells were stimulated with $2x10^5$ UV-irradiated MCMV-infected or uninfected BALB/c MEF and $2x10^7$ UV-irradiated influenza A/WSN-infected or uninfected P815, in a total volume of 200 ul and assayed for IL-3 release (Table 3). Release of IL-3 above the levels caused by uninfected control stimulator cells only occurred when virus-infected stimulator cells were added to immune T cells primed with the same virus, i.e., the immune T cells were virus-specific with respect to MCMV and influenza A/WSN.

Lyt phenotypes and H-2 restriction patterns of MCMV-immune LN cells that mediate cytotoxicity and release lymphokines

Previous experimentation revealed that the MCMV-immune LN cells generated according to the method described here were also cytotoxic to MCMV-infected MEF (5). Therefore, we investigated the phenotypic properties of the LN cell populations that were capable of lymphokine release or cytotoxicity.

A batch of BALB/c MCMV-immune LN cells was harvested and divided into three portions which were treated with C' alone, Lyt2 antiserum and C' or left untreated. Samples of each of the three portions were assayed for cytotoxicity on MCMV-infected or uninfected BALB/c MEF. A second sample of each of the three portions was suspended at $5x10^6$ cells/ml and 100 ul aliquots were removed and mixed with $2x10^5$ MCMV-infected or uninfected BALB/c MEF in a

total volume of 200 ul in a lymphokine release assay.

The results (Table 4) indicated that cytotoxicity against MCMV-infected BALB/c MEF and the ability to release IL-3 upon stimulation with MCMV-infected BALB/c MEF were both largely dependent upon MCMV-immune LN cells of $Lyt2^+$ phenotype. $Lyt2^-$ T cells were clearly not a prominent population in the assays used here.

Further characterization of MCMV-immune LN cells was performed by determining whether or not Class I H-2 antigens were capable of acting as the restriction element during assay for cytotoxic or lymphokine release functions. MCMV-immune LN cells from BALB/c and B10.AQR mice were harvested, pooled and then divided into equal portions such that each portion (1.2 ml) contained MCMV-immune LN cells obtained from 6 LN. For assessment of cytotoxic activity a 1/36 aliquot of the 6 LN equivalents were added to MCMV-infected or uninfected MEF target cells, whilst for lymphokine release assays $5x10^5$ MCMV-immune LN cells were mixed with $2x10^5$ MCMV-infected or uninfected MEF in a total volume of 200 ul. The infected or uninfected MEF used in both assays above were derived from BALB/c, CBA/H and B10.T(6R) mice.

Cytotoxic activity (Table 5) against MCMV-infected MEF was above levels obtained on uninfected MEF when B10.AQR MCMV-immune LN cells were reacted with BALB/c and B10.T(6R) targets, but this was not observed with CBA/H targets. In fact, there was decreased cytotoxicity on MCMV-infected CBA/H MEF in comparison with uninfected CBA/H MEF. IL-3 release (Table 5) followed a similar pattern to that of cytotoxic activity, except for the finding that similar amounts of IL-3 were released in reactions with either

MCMV-infected or uninfected CBA/H MEF. The positive results in the B10.AQR-BALB/c combination showed that D region Class I H-2 gene(s) code for restriction antigens involved in MCMV-specific T cell stimulation. Whether K region Class I H-2 genes are also involved has not yet been determined. The negative results for IL-3 release in the B10.AQR-CBA/H combination which shares Class II H-2 genes does not exclude the existence of Class II H-2-restricted anti-MCMV T cells, since MEF do not display Class II H-2 antigens (9).

The effect of time after MCMV-infection upon the ability of MEF to stimulate IL-3 release from MCMV-immune LN cells

Previous reports with ectromelia (10) and herpes simplex virus (HSV-1) (11) indicated that virus-induced antigenic changes recognised by cytotoxic T cells were expressed within 90 mins after viral adsorption. It was of interest to examine whether this was also the case for MCMV-infected MEF with respect to their ability to stimulate IL-3 release.

BALB/c MEF were adsorbed with MCMV as described in the Methods, and incubated for 0, 1, 2, 4, 6, 8, 16 or 23 hrs before trypsinization, UV irradiation and utilization at $2x10^5$ cells/100 ul to stimulate $5x10^5$ BALB/c MCMV-immune LN cells in a total volume of 200 ul. Uninfected BALB/c MEF stimulators were treated in a similar manner, except that virus was not adsorbed. Cell-free supernatants were harvested after 6 hrs and assayed for IL-3.

The results (Fig. 3) revealed maximum release of IL-3 from MCMV-immune LN cells when stimulated with syngeneic MEF infected

with MCMV for 8-24 hrs, but it seemed that there was antigen capable of stimulation by one hour post MCMV-infection, assuming that UV irradiation of the stimulator MEF arrested any further cell surface expression of MCMV antigens.

Comparison of ability of different numbers of 3 hrs or 18 hrs MCMV-infected BALB/c MEF to stimulate IL-3 release from syngeneic MCMV-immune LN cells

BALB/c MEF were infected with MCMV for either 3 hrs or 18 hrs prior to trypsinization and UV irradiation. These cells were resuspended at $2x10^7$ cells/ml, twofold dilutions performed, and $5x10^5$ MCMV-immune BALB/c LN cells added to a total volume of 200 ul. After 6 hrs incubation cell-free supernatants were collected and assayed for IL-3.

The results (Fig. 4) showed that for a fixed number of MCMV-immune LN cells, 18 hrs MCMV-infected BALB/c MEF, at all cell numbers, were capable of stimulating more IL-3 release than 3 hrs MCMV-infected BALB/c MEF. The amounts of IL-3 released for 18 hrs MCMV-infected BALB/c MEF reached a plateau at $2.5\text{-}5.0x10^5$ cells/well, whereas 3 hrs MCMV-infected BALB/c MEF peaked at $5x10^5$ cells/well. With uninfected BALB/c MEF stimulators $\log_{10}$ IL-3 titres were < 1.02.

DISCUSSION

It has been shown by other workers (12) that release of soluble T cell factors (generally termed lymphokines) from allo-antigen-activated T cells is triggered by an antigenic signal. We have shown here that MHC-restricted, antiviral T cells release the lymphokine, IL-3 (a multipotential growth factor for lympho-myeloid cells) when stimulated in vitro with syngeneic virus-infected MEF and have conducted a detailed investigation of this phenomenon.

Using MCMV-infection of mice as the model, it was shown that the release of IL-3 was dependent upon a Thy1.2$^+$, Lyt2$^+$ immune LN cell population and that antigen recognition by those T cells was MCMV-specific and Class I H-2 restricted when MCMV-infected MEF were used as stimulator cells (Tables 1-3). These data are consistent with, but do not prove, that the same T cells are capable of performing both cytotoxicity and IL-3 release functions. There is some precedent for this phenomenon in viral immunology, since anti-influenza A Lyt2$^+$ cytotoxic T cell clones are capable of cytotoxicity and release of the lymphokine, gamma-interferon, upon stimulation with virus-infected MHC-matched stimulator cells (13). Proof that the same MCMV-immune T cells are capable of both functions awaits establishment of anti-MCMV cytotoxic T cell clones. Nevertheless, these investigations emphasize further that the Lyt2$^+$ phenotype does not necessarily correlate only with the specific function of cytotoxicity as originally suggested by Cantor and Boyse (14).

The H-2 restriction pattern clearly indicated that H-2D^{d} acted as a restriction element for MCMV-immune T cells but we have not yet investigated H-2K antigens. Because MEF do not possess Class II antigens on the cell surface, these current experiments do not investigate the presence or absence of Class II-restricted MCMV-immune T cells. Data suggesting that Class II MHC-restricted Lyt2^{-} anti-MCMV T cells were also present in immune LN cell populations will be the subject of a future report.

Currently we are attempting to obtain cell populations that express Class II MHC and MCMV-specific antigens for use as stimulators of IL-3 release or targets for cytotoxicity.

With a fixed number of MCMV-immune T cells, stimulation with increasing numbers of MCMV-infected MEF caused a linear increase in IL-3 titres, plotted on log-log scale. It is noteworthy that this curve is typical of a single hit relationship between two interacting species. As IL-3 release is almost certainly dependent upon T cell-stimulator cell conjugate formation the curve can be easily explained by assuming that one stimulator cell is sufficient to trigger IL-3 release from one cell. Thus the early region of the curve represents T cell excess and IL-3 titre is directly proportional to the number of stimulator cells added whilst the plateau results from the maximum stimulation of all antigen-reactive T cells. This simple behaviour is not always the case as some alloreactive T cell-stimulator cell combinations display more complex kinetic characteristics (P. Hodgkin, unpublished results). The reasons for the decreased IL-3 titre produced at high MCMV-infected MEF numbers is unclear, but may reflect _in vitro_ artefact rather than the release of

inhibitors during the 6 hrs assay period, because no evidence for inhibitor production was seen for up to 24 hrs of interaction between T and stimulator cells (Fig. 2).

The effect of different time intervals between infection of MEF with MCMV and their utilization as stimulators of MCMV-immune T cells is of interest. The results indicated that as time after MEF infection was increased, the capacity to stimulate IL-3 release from MCMV-immune T cells was increased (Fig. 3). Three possible explanations for this phenomenon are proposed. 1. The rate of lymphokine release can vary with the amount of antigenic stimulus received by the T cell. As antigen per stimulator cell increases the rate of IL-3 release is increased. 2. The MCMV-immune T cell population is heterogeneous with a proportion of cells capable of recognising "early" expressed MCMV antigens. At later times the cummulative effect of T cell stimulation by all MCMV antigens is seen. 3. At early times less MEF are expressing MCMV antigen and therefore more 3 hrs MCMV-infected stimulators are required to achieve equivalence with the number of stimulators present in the 18 hrs MCMV-infected MEF population. Theoretically it is possible to distinguish model 1 and 2 from model 3 from a study of the antigen dose-response characteristics. Models 1 and 2 predict that in the plateau region of antigen excess a difference will be seen between 3 hrs and 18 hrs MCMV-infected stimulator induced lymphokine release. For model 1 this difference would reflect the different rate of lymphokine release and for model 2 it would indicate the proportion of "early"-antigen reactive T cells versus the total number of T cells triggered by MCMV antigens. Model 3 on the other hand predicts that 3 hrs MCMV-

infected stimulators when added in excess will reach the same plateau level as for 18 hrs MCMV-infected stimulators. The results (Fig. 4) indicated the attainment of different plateaux which argue against model 3. Further investigation is required to distinguish between models 1 and 2.

The relevance of these *in vitro* findings to the *in vivo* immunobiology of MCMV and other viral infections requires further study. Until the recent report of IL-3 gene cloning (15), this product was obtained in small amounts of variable purity from the myeloid leukemic cell line WEHI-3B (16), lectin-activated T cells (17), alloantigen-activated T cells (18) or viral antigen-activated T cells (19). This has limited investigations of *in vivo* function and therefore specific questions related to IL-3 release from virus-immune T cell populations and its effects upon haemopoietic function and immunobiology of various viral infections awaits an understanding of the general biology of this glycoprotein. Nevertheless, the role of IL-3 as a member of a class of glycoproteins known as CSF has been well established *in vitro*. It stimulates a wide range of lymphoreticular stem cells and some of their more differentiated progeny, and consequently on the basis of these biological activities, has been given several names which include burst-promoting activity (20), P-cell-stimulating factor (21), mast cell growth factor (22), haemopoietic cell growth factor (23), histamine-producing cell-stimulating factor (24) and multicolony-stimulating factor CSF (25).

Concanavalin-A-stimulated T cell hybridomas are capable of liberating more than one lymphokine (26). Therefore it would seem important to investigate the spectrum of lymphokines (e.g., gamma-

interferon, IL-2, B cell growth factor) produced by antiviral T cell populations (and clones) of different Lyt phenotypes and different MHC restriction patterns in different model infections.

SUMMARY

MCMV-immune LN cells were generated following 4 days culture of draining popliteal LN cells removed after bilateral hind f.p. inoculation of mice with $4x10^2$ p.f.u. MCMV. This cell population expressed both cytotoxic activity against MCMV-infected MEF and the capacity to release lymphokine upon stimulation with MCMV-infected MEF. Cytotoxicity and lymphokine release were Class I, H-2 restricted, viral specific and dependent upon $Thy1.2^+$, $Lyt2^+$ cells.

Mixing of $5x10^5$ MCMV-immune T cells with an excess of syngeneic MCMV-infected MEF stimulators produced detectable levels of the lymphokine, IL-3, by 1 hr, a maximum rate of IL-3 release between 1-6 hrs and plateau levels by 14 hrs.

The capacity to stimulate virus-specific MCMV-immune T cells was acquired by MEF at 1 hr post MCMV-infection with maximum stimulator ability attained by 8 hrs. In the presence of a fixed number of MCMV-immune T cells, and titration of syngeneic 3 hrs or 18 hrs MCMV-infected MEF to excess, lower IL-3 plateau levels were obtained with 3 hrs than with 18 hrs MCMV-infected stimulators. This suggested that fewer T cells responded to 3 hrs than to 18 hrs MCMV-infected MEF.

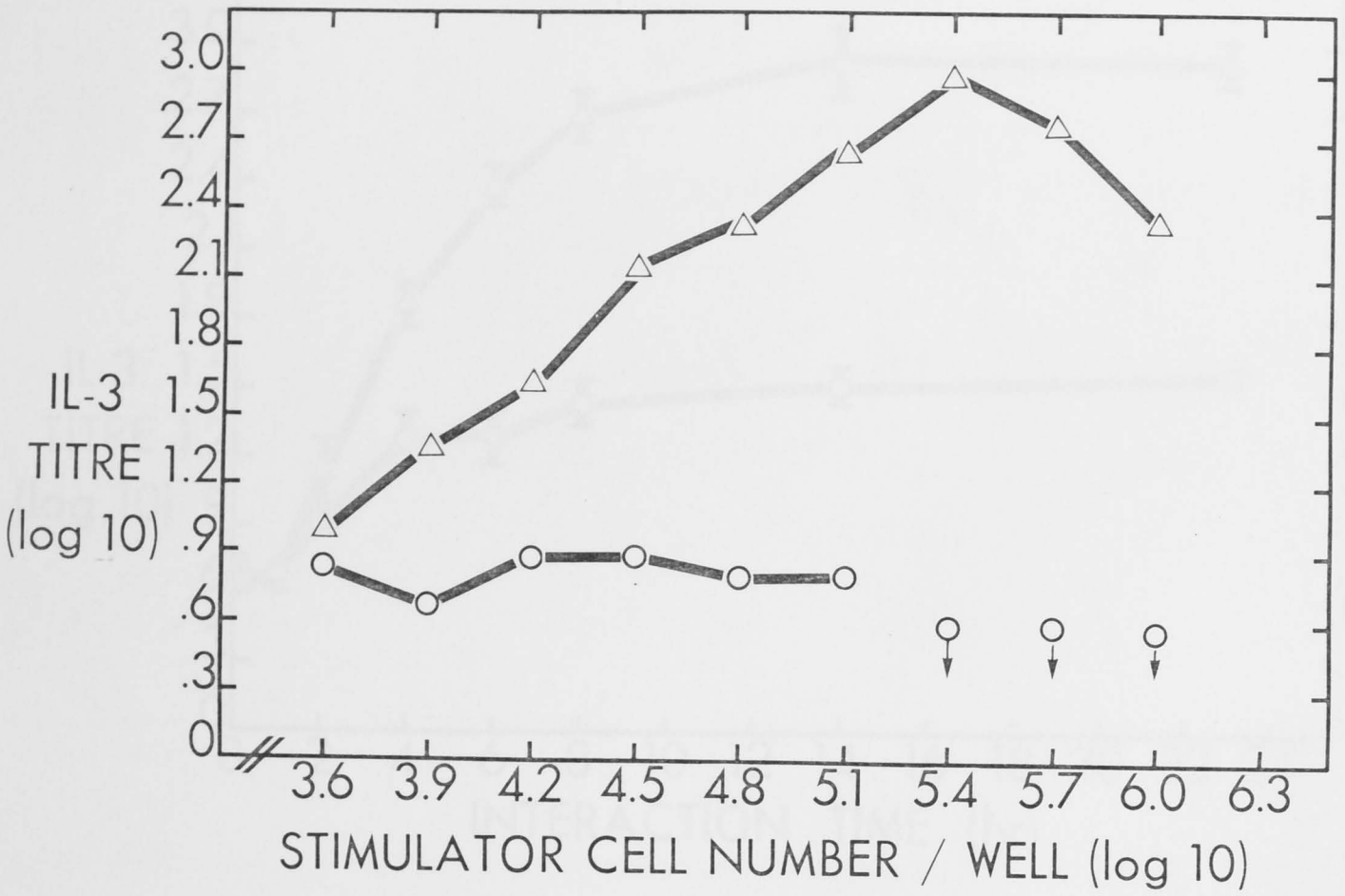

Fig. 1.
Curve showing the increase in IL-3 release from $5x10^5$ B10.AQR, MCMV-immune LN cells caused by increasing numbers of MCMV-infected (△) or uninfected (○) syngeneic MEF stimulator cells. Each point represents the mean of quadruplicates ± S.D. of the IL-3 titre, for supernatants harvested after 6 hrs at 37°C. (○↓) IL-3 titre was below detection levels. S.D. are so small that they cannot be seen outside the points.

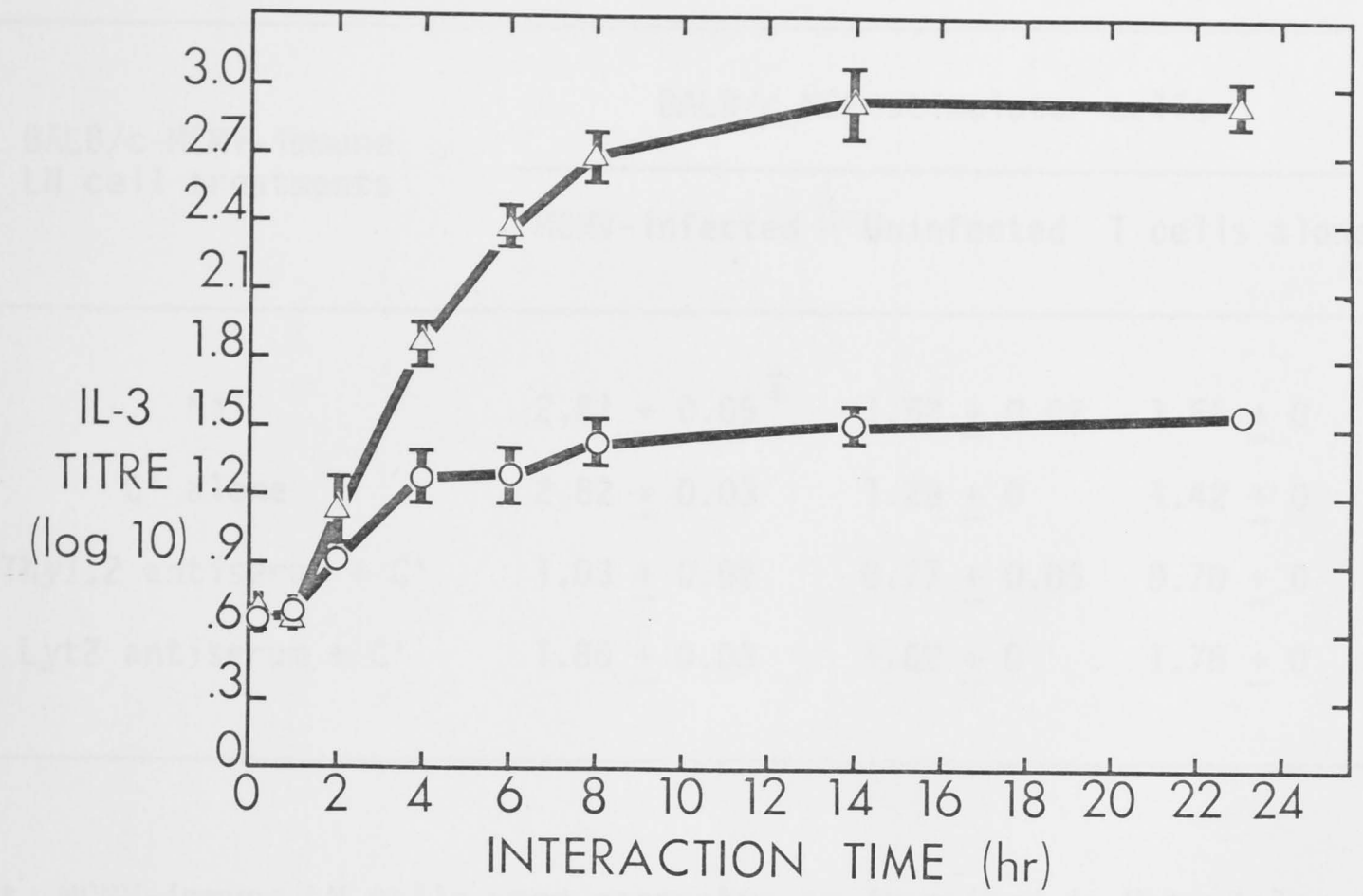

Fig. 2.
Kinetics of IL-3 release from 5×10^5 BALB/c MCMV-immune LN cells stimulated with 2×10^5 MCMV-infected (△) or uninfected (○) syngeneic MEF. Each point represents the mean of quadruplicates ± S.D. of the IL-3 titre for supernatants harvested after the interaction times indicated. Where no S.D. bars are shown the deviations were not outside the points.

TABLE 1

Effect of Thyl.2 and Lyt2 antiserum plus C' treatment of MCMV-immune LN cells* upon their ability to release IL-3

BALB/c MCMV-immune LN cell treatments	BALB/c MEF stimulator cells		
	MCMV-infected	Uninfected	T cells alone§
Nil	2.81 ± 0.05‡	1.52 ± 0.02	1.66 ± 0
C' alone	2.82 ± 0.03	1.29 ± 0	1.42 ± 0
Thyl.2 antiserum + C'	1.03 ± 0.02	0.77 ± 0.05	0.70 ± 0
Lyt2 antiserum + C'	1.86 ± 0.03	1.62 ± 0	1.78 ± 0

* MCMV-immune LN cells were generated as described in Materials and Methods.

‡ Log_{10} IL-3 titre from $5x10^5$ MCMV-immune LN cells added to $2x10^5$ MCMV-infected or uninfected MEF for 6 hrs at 37°C. Data given are the means of quadruplicates ± S.D.

§ $5x10^5$ BALB/c MCMV-immune LN cells were suspended in 200 ul without syngeneic MCMV -infected or uninfected MEF for 6 hrs at 37°C.

TABLE 2

H-2 restriction of stimulation of IL-3 release from MCMV-immune LN cells*

MCMV-immune LN cells	MEF stimulator cells (H-2 map)†			
	CBA/H (kkkkkkk)		B10.&(6R) (qqqqqqd)	
	MEF-infected	Uninfected	MCMV-infected	Uninfected
CBA/H	2.19 ± 0.03 ‡	1.40 ± 0.02	1.55 ± 0.02	1.47 ± 0
B10.T(6R)	2.15 ± 0.02	2.24 ± 0.05	2.60 ± 0.05	1.04 ± 0.03

* ‡ As for Table 1

† H-2 map for K, A_α, A_β, E_β, J, E_α and D genetic regions.

TABLE 3

Viral specificity of stimulation of IL-3 release from MCMV- or Influenza-immune T cells

Virus-immune* T cells	Stimulator Cells			
	MCMV-infected BALB/c MEF	Uninfected BALB/c MEF	A/WSN-infected P815	Uninfected P815
MCMV-immune LN cells	2.64 ± 0.03‡	0.94 ± 0.04	1.20 ± 0.03	1.13 ± 0.03
Influenza A/WSN-immune spleen cells	1.03 ± 0.02	1.20 ± 0.02	2.43 ± 0	1.20 ± 0.06

* MCMV-immune LN cells were generated as in Table 1. Influenza A/WSN-immune cells were obtained by *in vitro* restimulation of influenza A/WSN-primed spleen cells (Yap & Ada, 1977).

‡ As for Table 1.

TABLE 4

Effect of Lyt2 antiserum and C' treatment upon cytotoxicity and IL-3 release mediated by MCMV-immune LN cells*

BALB/c MCMV-immune LN cell treatments	% Lysis§		IL-3 Titre‡	
	BALB/c MEF targets		BALB/c MEF stimulator cells	
	MCMV-infected	Uninfected	MCMV-infected	Uninfected
Nil	72.5 ± 2.9	17.8 ± 0.5	2.03 ± 0.11	0.72 ± 0
C' alone	61.4 ± 0.5	9.9 ± 0.3	2.19 ± 0.08	0.60 ± 0
Lyt2 + C'	11.3 ± 0.5	6.0 ± 0.2	0.93 ± 0	0.60 ± 0

* ‡ As for Table 1

§ The % lysis values are the means ± standard errors of the mean of 3 replicates obtained by the addition of a 1/36 aliquot of cultured cells, obtained from 6 BALB/c popliteal LN, onto targets.

TABLE 5

H-2 restriction pattern of the cytotoxic and IL-3 release functions of target and stimulator cells

Strain of MCMV-immune LN cells* (H-2 map)†	Effector function	BALB/c MEF (d d d d d d d)		CBA/H MEF (k k k k k k k)		B10.T(6R) MEF (q q q q q q d)	
		MCMV-infected	Uninfected	MCMV-infected	Uninfected	MCMV-infected	Uninfected
BALB/c (d d d d d d d)	% lysis§	71.0 ± 3.9	11.4 ± 1.5	-	-	-	-
	IL-3 titre ‡	3.34 ± 0.04	1.28 ± 0.02	2.39 ± 0.11	2.19 ± 0.04	2.81 ± 0.06	1.47 ± 0
B10.AQR (q k k k k k d)	% lysis	65.5 ± 1.1	28.4 ± 6.9	38.0 ± 1.8	67.0 ± 0.8	52.7 ± 2.6	14.9 ± 0.2
	IL-3 titre	3.3 ± 0	1.59 ± 0.04	2.81 ± 0.02	2.75 ± 0.02	2.93 ± 0.02	0.87 ± 0

* ‡ † As for Tables 1 and 2.

§ % lysis values as for Table 4.

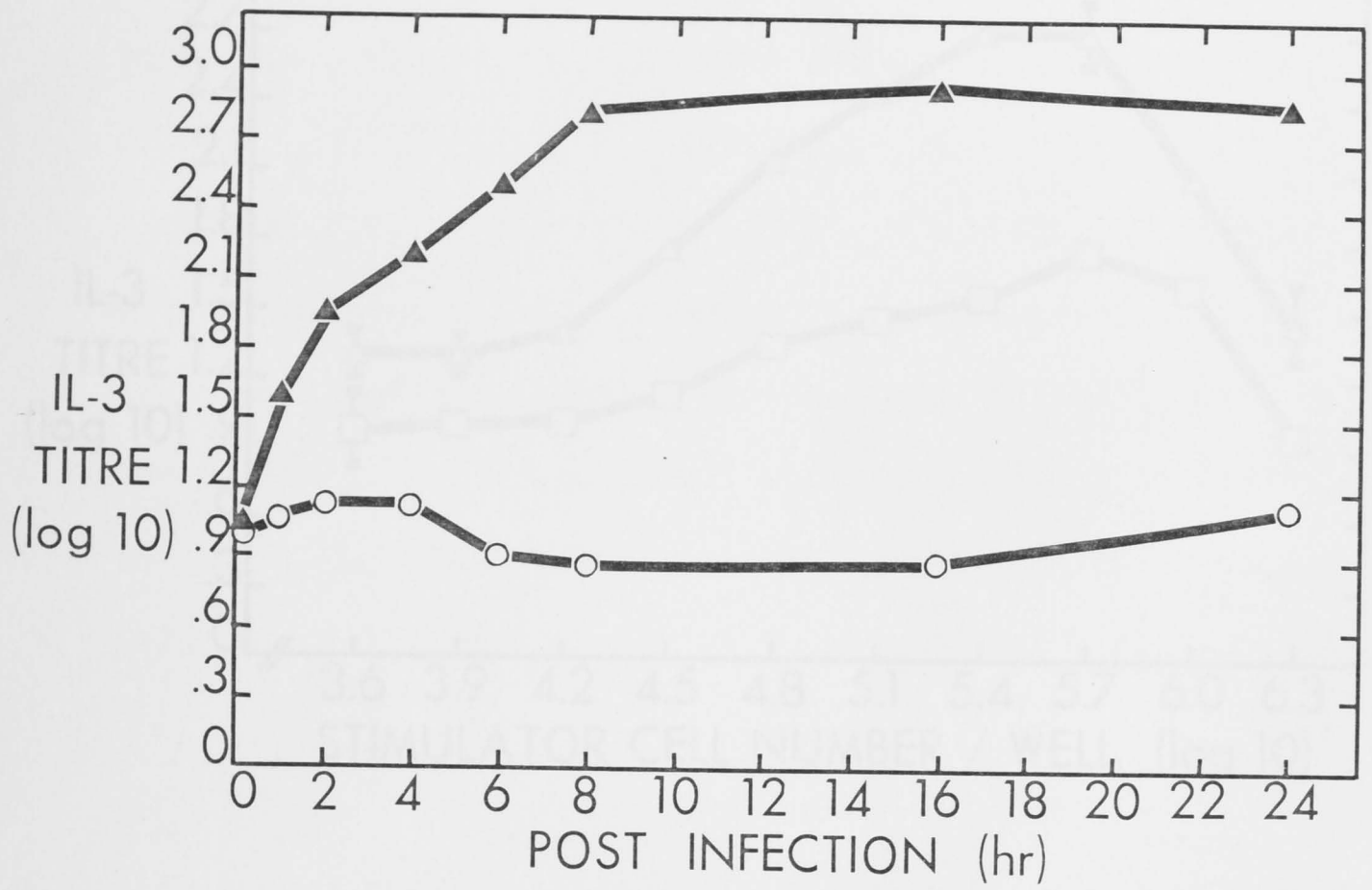

Fig. 3.
Curve indicating IL-3 release from $5x10^5$ BALB/c MCMV-immune LN cells when stimulated with $2x10^5$ syngeneic MEF infected with MCMV for the times indicated (△). Uninfected syngeneic MEF stimulators (○). Each point represents the mean of quadruplicates ± S.D. of the IL-3 titre for supernatants harvested after 6 hrs at 37°C. S.D. are so small that they cannot be seen outside the points.

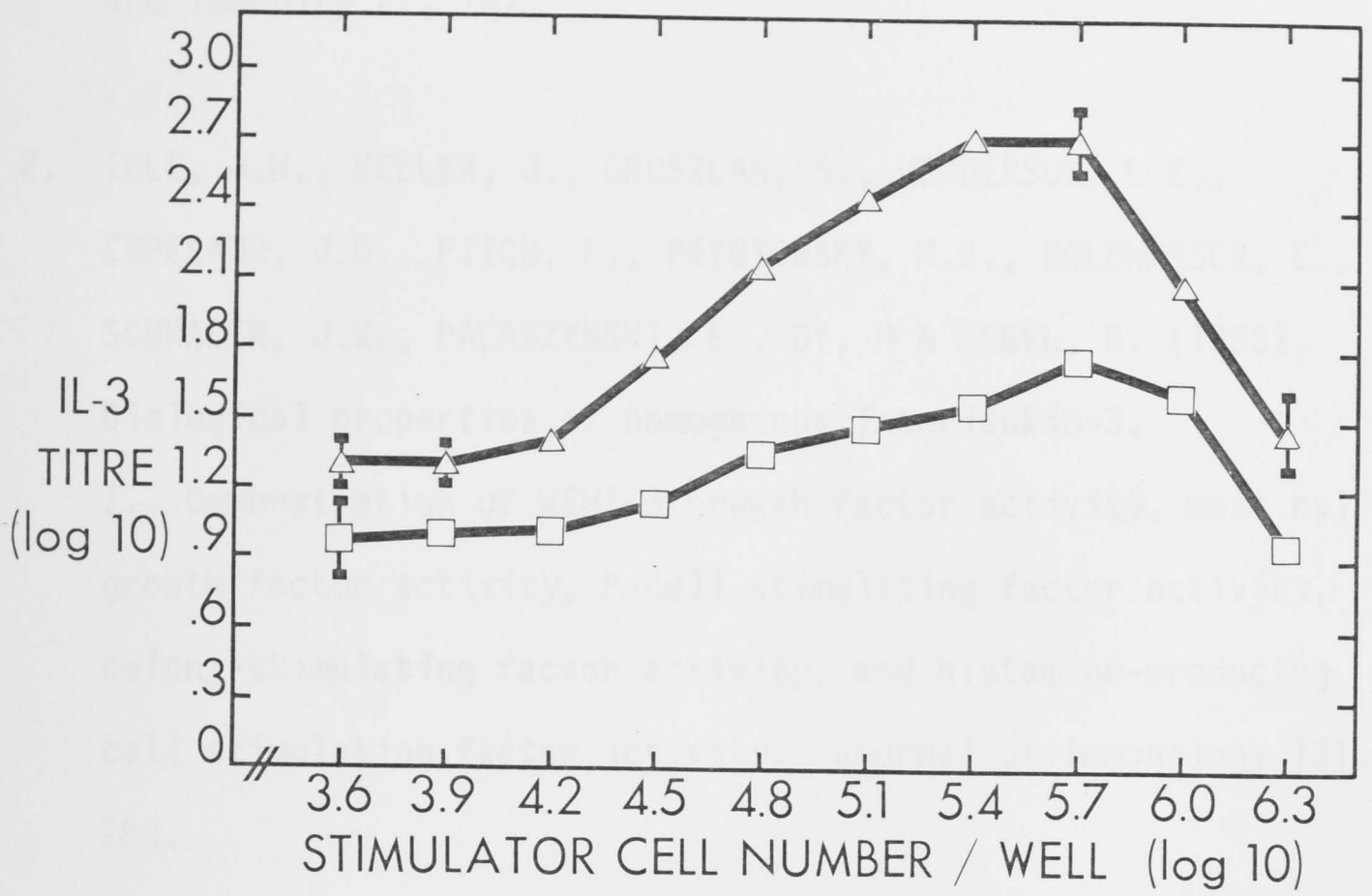

Fig. 4.
Curve showing IL-3 release from 5×10^5 BALB/c MCMV-immune LN cells produced by increasing numbers of MCMV-infected syngeneic MEF infected for 18 hrs (△) or 3 hrs (□) prior to use as stimulators. Uninfected syngeneic MEF are not shown for reasons of clarity but IL-3 titres were 1.02. Each point represents the mean of quadruplicates ± S.D. of the IL-3 titre for supernatants harvested after 6 hrs at 37^{o}C. Where no S.D. bars are indicated the deviations were not outside the points.

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CHAPTER 5

The Cytotoxic Response to Murine Cytomegalovirus (MCMV).

IV. Requirements for the generation of MCMV-specific target cells

INTRODUCTION

It has been well-defined that T cells are important in recovery from acute viral infections (Blanden 1974, Ada et al. 1981) and that these T cells are both H-2 restricted and virus specific (Kees & Blanden 1976). As an approach to the immunobiology of viruses that cause latent infections, the T cell response to murine cytomegalovirus (MCMV) was investigated. Previous reports (Sinickas et al. in press) showed that hind footpad inoculation of mice with MCMV, removal of popliteal lymph node (LN) cells at day 7 and subsequent culture of these LN cells for 4 days, was a reliable method for the generation of MCMV-immune T cells. One functional characteristic of this immune-T cell population, cytotoxicity, was then utilised to study the requirements for generation of MCMV-specific target cells.

MATERIALS AND METHODS

Mice. BALB/c, C57Bl/6J and WEHI-3 mice, 6-12 weeks of age were obtained from the Animal Breeding Establishment of the John Curtin School of Medical Research.

Viruses. Standard methods were used for the preparation of salivary gland stock of the Smith strain of MCMV (Sinickas et al. 1985, in press). Tissue culture passaged Smith strain MCMV was prepared by modification of a described method (Ebeling et al. 1983). Briefly, secondary mouse embryo fibroblasts (MEF) were seeded into 30 cm^2 plastic petri dishes (Cat. No. P.D.S. 6515H, Kayline Plastics, Thebarton, S.A., Australia) at $10^{6.0}$ cells in 10 ml of Dulbecco's Modified Eagle's Medium (Cat. No. H16, GIBCO, Grand Island, N.Y., U.S.A.)supplemented with 5% foetal calf serum (FCS) (Flow Labs, Stanmore, N.S.W., Australia), 200 ug/ml Streptomycin, 200 U/ml Penicillin G and 125 ug/ml Neomycin sulphate (antibiotics) (the complete medium is referred to hereafter as DMEM) in an atmosphere of 10% CO_2, 7% O_2 and 83% N_2 (special gas) (Commonwealth Industrial Gases, Alexandria, N.S.W., Australia) and infected 20 hrs later with 0.5 plaque forming units (p.f.u.)/ cell of salivary gland stock MCMV in 0.8 ml. The adsorption protocol involved centrifugation at 800g for 20 mins at room temperature and an additional 40 mins at 37^{o}C in special gas. Subsequently the cells were trypsinised and added to trypsinised uninfected secondary MEF at a ratio of 1:100. This cell suspension was seeded at $10^{7.18}$ cells/60 ml DMEM into 175 cm^2 tissue culture flasks (Nunc, Roskilde, Denmark), and incubated at 37^{o}C in

special gas. Two days later cytopathic effects were seen in monolayers and after 3 days all the cells were detached from the tissue culture flask. The cells and culture fluid were collected and centrifuged at 22,000 rpm for 2 hrs at 4^{0}C (Beckman SW27 rotor). The pellet was resuspended in DMEM at 1/20 of the original volume and sonicated for 60 secs at 50 cps (Sonifier B12, Branson Sonic Power Company, Danbury, Conn., U.S.A.). All aliquots were stored at -70^{0}C.

Concanavalin-A-stimulated spleen cell supernatant (CSS). This was prepared as previously described (Sinickas et al. 1985, in press) using spleen cells from WEHI-3 mice.

Cell culture. Mouse embryo fibroblasts and the continuous cell line BALB/c-3T3 were cultured as previously described (Sinickas et al. 1985, in press). The DBA/2 mastocytoma cell line P-815-X2 (P815) was cultured in DMEM at 37^{0}C in special gas atmosphere. Similar conditions were utilised for the murine macrophage cell line PU5-1.8, except that the medium was supplemented with 10^{-4}M asparagine. The Interleukin-3 (IL-3) dependent cell line, FDC-P1, was grown in RPMI-1640 (Cat. No. 10-601-20, Flow Labs Inc., Maclean, Virginia, U.S.A.) supplemented with 5% heat-inactivated FCS (HI-FCS) and 10% WEHI-3B cell line-conditioned medium (kindly provided by Dr. A.J. Hapel).

Cytotoxicity assays. Peritoneal cells (PEC) were harvested using 10 ml ice-cold Puck's saline from mice inoculated intra-peritoneally with 2 ml of 3% wt/vol thioglycollate medium

(DIFCO Labs, Michigan, U.S.A.) 5 days previously. The cells were centrifuged at 600g for 5 mins and resuspended in Eagle's Minimal Essential Medium (EMEM) (Cat. No. F15, GIBCO, Grand Island, N.Y., U.S.A.) supplemented with 10% FCS and antibiotics (Assay medium) at the required cell concentration. After 60 mins viral adsorption at 37^{0}C in 5% CO_2, 600 uCi $Na_2[^{51}Cr]O_4$ (^{51}Cr) (Amersham Int. Ltd., Amersham, U.K.) in 1 ml of assay medium was added and the cells reincubated for a further 60 mins. The cells were then washed twice with assay medium, reincubated at 37^{0}C in 5% CO_2 for 60 mins, washed again, concentration adjusted to $10^{5.3}$ cells/ml and 100 ul aliquots dispensed into flat-bottomed 96 well trays (Nunc, Roskilde, Denmark). Subsequently, 100 ul of MCMV-immune LN cells were added.

For P815 target cells, $10^{6.0}$ cells were infected with MCMV at a multiplicity of infection (MOI) of 5 p.f.u./cell in a total volume of 1.4 ml DMEM at 37^{0}C in special gas for 60 mins. Subsequently 3.6 ml DMEM containing 6 uCi/ml of ^{51}Cr was added and incubation continued under conditions described for PEC. After 16 hrs the cells were washed twice with DMEM and resuspended at $10^{5.3}$ cells/ml. 100 ul aliquots of cell suspension were added to 96-well round-bottomed microtitre trays followed by 100 ul of MCMV-immune LN cells.

For PU5-1.8 target cells, $10^{5.7}$ cells were adsorbed with MCMV at a MOI of 5 p.f.u./cell in a total volume of 600 ul DMEM at 37^{0}C in special gas for 60 mins. Subsequently 2.5 ml of DMEM containing 6 uCi/ml of ^{51}Cr was added and incubation continued for 16 hrs. All further manipulations were as for P815 cells.

For MEF and BALB/c-3T3 targets the protocol was usually unchanged from that described previously (Sinickas et al. 1985,

in press).

Where the infection protocol was altered the changes are described in the Results section. ^{51}Cr release from the targets was assayed, unless otherwise stated, after a 10 hrs incubation at $37^{O}C$ by gamma emissions and lysis calculated using the following formula:

$$\% \text{ lysis of infected or uninfected targets} = \frac{^{51}Cr \text{ counts in presence of effector cells} - ^{51}Cr \text{ counts released in medium}}{^{51}Cr \text{ counts water-lysed targets} - ^{51}Cr \text{ counts released in medium}}$$

% lysis = % lysis infected targets - % lysis uninfected targets.

Generation of antiviral and alloreactive T cells.

A previously described protocol for MCMV-immune T cell (Sinickas et al. 1985, in press) generation was used. Briefly, BALB/c female mice were inoculated into both hind footpads (f.p.) with either $10^{2.6}$ or $10^{4.6}$ p.f.u. MCMV. At 7 days post-inoculation the draining popliteal lymph node (LN) cells were removed and cultured for 4 days. Subsequently, these cells were harvested, pooled and divided into portions equivalent to the cells obtained from 6 MCMV-stimulated LN and added to different target cells in cytotoxicity assays.

Alloreactive T cells were generated according to a published method (Woolnough & Lafferty 1979).

Preparation of stimulator cells for lymphokine production.

Secondary MEF were subcultured into 30 cm^2 plastic dishes (Cat. No. P.D.S. 6515H, Kayline Plastics, Thebarton, S.A., Australia) at 10^6 ml DMEM, supplemented with or without 3% vol/vol CSS/dish. The cells were incubated for 2 days at 37^oC in special gas, washed, fresh medium without CSS added and 16 hrs later, trypsinized and resuspended in DMEM at 10^6 cells/ml. Ten ml aliquots were then added to 65 cm^2 plastic petri dishes (Cat. No. 992550, Medical Plastics, Edwardstown, S.A., Australia) and subjected to ultraviolet (UV) irradiation from an 80 watt germicidal lamp for 4 mins at an intensity of 960 uW/cm^2 in the 230/270 nm range. These cells were then washed twice and resuspended at an appropriate concentration in EMEM supplemented with 10^{-4}M 2-mercaptoethanol (2ME). Aliquots of 0.1 ml were dispensed into 96-well flat-bottomed trays (Nunc, Roskilde, Denmark). Serial twofold dilutions were prepared prior to addition of alloreative T cells.

Conditions for stimulation of lymphokine production.

Alloreactive T cells were harvested, washed twice and resuspended in EMEM supplemented with 10^{-4}M 2ME at $5x10^6$ cells/ml. 100 ul aliquots were dispensed into 96-well flat-bottomed plates containing 100 ul aliquots of CSS treated or untreated stimulator MEF cells. Duplicates of these cell mixtures were incubated at 37^oC in special gas mixture for 6 hrs, after which time cell-free supernatants were removed and stored at -20 C until assayed for IL-3.

IL-3 assay. IL-3 activity was assayed by measuring the ability of lymphokine preparations to maintain in vitro proliferation of the IL-3-dependent FDC-P1 cell line. After a period of 16-24 hrs without IL-3, PDC-P1 cells cease dividing. Serial twofold dilutions of the test supernatants were prepared in 96-well flat-bottomed plates (Linbro Cat. No. 76-203-05, Flow Labs Inc., MacLean, Virginia, U.S.A.) with 50 ul RPMI-1640 plus 5% heat-inactivated FCS (HI-FCS) as diluent. Subsequently, 50 ul of a FDC-P1 cell suspension ($4x10^5$) was added to each culture well. Control preparations consisted of 48 replicate cultures containing 50 ul of the FDC-P1 cell suspension and 50 ul of RPMI supplemented with 5% HI-FCS. The cultures were incubated at 37^oC for 16-20 hrs in 5% CO_2, after which time 50 ul of [Methyl-^{3}H]-thymidine (Amersham Int. Ltd., Amersham, U.K.) (25 uCi/ml) were added and incubation continued for a further 5 hrs. [Methyl-^{3}H]-thymidine incorporation was then determined by harvesting the cells onto glass fibre paper using a MASH II multiple sample harvester (Microbiological Associates, Bethesda, Md., U.S.A.). The dried samples of glass fibre paper were placed into 7 ml of scintillation fluid and beta-emission counted in a liquid scintillation counter (Tricarb, Packard Instrument Co., Downers Grove, Ill., U.S.A.). All samples were assayed in duplicate. The titration endpoint was set at three standard deviations above the mean control level of [Methyl-^{3}H]-thymidine uptake, according to a previously described method (Lafferty et al. 1980).

RESULTS

Susceptibility of different H-2^d MCMV-treated target cells to lysis by BALB/c (H-2^d) MCMV-immune T cells

A batch of MCMV-immune T cells were harvested and aliquots added to different target cells in cytotoxicity assays. Two experiments were conducted in which BALB/c MCMV-infected MEF were used as positive controls in comparison with MCMV-treated and untreated H-2^d haplotype P815, PU5-1.8 and BALB/c-3T3 cells.

Under the conditions used MCMV-treated BALB/c MEF were the targets most susceptible to lysis by MCMV-immune T cells over and above untreated control target cells (Table 1).

Ways of improving the peformance of MEF as target cells were then investigated, based on the knowledge that expression of both host cell H-2 antigens and viral antigens on infected MEF are required for T cell recognition.

The effect of BALB/c MEF culture in CSS-supplemented medium upon susceptibility to lysis by alloreactive T cells

γ-interferon, known to be present in CSS, can increase H-2 antigen expression on the cell surface (Wong et al. 1983) thus increasing the sensitivity of the cell to recognition and lysis by T cells (O'Neill & Blanden 1979). BALB/c MEF were grown in DMEM supplemented with or without 3% vol/vol CSS for 48 hrs, then washed with DMEM and incubated for a further 16 hrs prior to utilisation as targets in cytotoxicity assays using C57Bl/6J-

anti-BALB/c T cells.

Exposure to CSS significantly enhanced the susceptibility of target cells to lysis by alloreactive cytotoxic T cells, at all effector:target ratios (Table 2).

Effect of BALB/c MEF culture in CSS-supplemented medium upon the ability to stimulate IL-3 release from alloreactive T cells

BALB/c MEF were grown in DMEM supplemented with or without 3% vol/vol CSS for 48 hrs, then washed with DMEM and incubated for a further 16 hrs prior to utilisation as stimulator cells in IL-3 assays. $10^{5.7}$ C57Bl/6J-anti-BALB/c T cells were mixed with different numbers of CSS-treated or untreated BALB/c MEF, starting at $10^{4.2}$ cells with 0.3(log 10) increments up to $10^{6.3}$ cells in a total volume of 0.2 ml and incubated at 37°C in special gas for 6 hrs. Subsequently, cell-free supernatants were removed and assayed for IL-3.

The results (Fig. 1) showed that CSS-treated BALB/c MEF stimulated higher maximum levels of IL-3 from a fixed number of alloreactive T cells than did untreated BALB/c MEF. Fewer CSS-treated BALB/c MEF stimulators than untreated MEF were required to stimulate the same IL-3 titre. Also, the increase in IL-3 titre per unit increase in the number of BALB/c MEF stimulators was larger with CSS-treated BALB/c MEF than with untreated BALB/c MEF.

Taken together the data in Table 2 and Fig. 1 strongly suggest that CSS treatment of MEF enhances their recognition by T cells.

Effect of centrifugation during viral adsorption upon susceptibility of MCMV-infected BALB/c MEF target cells to lysis by syngeneic MCMV-immune T cells

Tertiary MEF cultured in CSS supplemented DMEM were adsorbed with 25 ul of salivary gland stock of MCMV diluted 1:8 in DMEM, with or without concomitant centrifugation at 800g for 20 mins at room temperature. A further 40 mins incubation at $37^{O}C$ in special gas completed the adsorption protocol. In cytotoxicity assays, aliquots of MCMV-immune T cells were mixed with syngeneic 16 hrs, MCMV-infected MEF. For uninfected MEF targets the protocols were similar except that 25 ul DMEM was added during the adsorption phase.

Centrifugation of MEF and MCMV suspension during the viral adsorption phase significantly enhanced susceptibility to lysis of MCMV-infected BALB/c MEF (Table 3).

The effect of CSS supplemented culture medium upon susceptibility of MCMV-infected or uninfected BALB/c MEF to lysis by syngeneic MCMV-immune T cells

Tertiary MEF were cultured in DMEM supplemented with or without 3% vol/vol CSS for 2 days prior to infection using the centrifugation protocol with 1:8, 1:16 and 1:32 dilution of salivary gland MCMV stock in DMEM. Sixteen hours after adsorption MCMV-infected MEF targets were mixed with aliquots of MCMV-immune LN cells. Uninfected MEF were treated in a similar manner except for lack of viral adsorption.

The results (Fig. 2) showed that lysis of MEF cultured with CSS was greater than untreated MEF provided the MCMV stock was used at 1:8 or 1:16 dilutions.

Kinetics of ^{51}Cr release from MCMV-infected and uninfected BALB/c MEF after addition of syngeneic MCMV-immune T cells

Aliquots of BALB/c MCMV-immune T cells were added to MCMV-infected or uninfected syngeneic MEF. Twenty-five ul aliquots of cell-free supernatant were removed 0, 2, 4, 6 and 10 hrs after mixing of MCMV-immune T cells and MEF targets.

The results (Fig. 3) showed the rate of ^{51}Cr release from MCMV-infected MEF was directly proportional to the number of MCMV-immune T cells added. The maximum rate of ^{51}Cr release was achieved using either 1/12 or 1/36 fraction of cultured LN cells. The kinetics of ^{51}Cr release for cytotoxicity obtained with uninfected MEF also revealed that the rate of ^{51}Cr release was directly proportional to the number of MCMV-immune T cells added to uninfected MEF. By 10 hrs, lysis of uninfected MEF was substantial at the higher effector:target ratios, and threatening to overtake lysis of infected MEF. Therefore, 10 hrs assays were used for MEF.

Events contributing to ^{51}Cr release over the 10 hrs interval include (a) development of virus-induced antigenic changes on the surface membrane of MEF; (b) contact between target MEF and effector T cells; (c) lysis of the target leading to ^{51}Cr release. The contribution of (a) above was examined further.

The effect of time after MCMV adsorption upon susceptibility of MCMV-infected BALB/c MEF to lysis by syngeneic MCMV-immune T cells

BALB/c MEF cultured in CSS-supplemented DMEM were washed and adsorbed with 25 ul salivary gland stock of MCMV diluted 1:8 in DMEM containing ^{51}Cr using the centrifugation protocol. At 0, 2, 4, 18 or 24 hrs after adsorption, the MEF were washed and 100 ul of MCMV-immune T cells added. The reaction vessel was then centrifuged for 1 minute at 800g to promote rapid contact between effector and target cells and incubated for 45 mins at $37^{0}C$ in special gas to allow target lysis to be initiated. Subsequently, 100 ul of DMEM supplemented with 0.04M EDTA was added to stop effector cell action, incubation continued at $37^{0}C$ in special gas and after 4 hrs, 100 ul of cell-free supernatant removed and assayed for gamma emissions. A control assay was performed in which 100 ul of MCMV-immune T cells were mixed with 18 hrs MCMV-infected BALB/c MEF and 100 ul of DMEM supplemented with 0.04M EDTA added immediately. The remainder of the assay was performed as described. All infection times were calculated from the end of the viral adsorption phase and up to the time of MCMV-immune T cell addition; but do not include the 4 hrs ^{51}Cr release phase.

The results showed that BALB/c MEF infected with MCMV for 2 hrs were susceptible to lysis by MCMV-immune T cells (Table 4). Susceptibility was increased by 4 hrs and was not significantly higher by 18 hrs post MCMV infection. Immediate addition of DMEM supplemented with 0.04M EDTA to the centrifuged mixture of MCMV-immune T cells and 18 hrs MCMV-infected BALB/c markedly diminished the amount of ^{51}Cr released thus confirming the efficiency of EDTA in terminating effector T cell action.

Susceptibility of BALB/c MEF and PEC infected with salivary gland stock MCMV to lysis by syngeneic MCMV-immune T cells

Since PEC have been successfully used as target cells with other viruses (Zinkernagel & Doherty 1975), they were investigated in the present series of experiments. $10^{5.7}$ BALB/c PEC in 300 ul assay medium were added to 300 ul of 1/4, 1/8, 1/16 or 1/32 dilutions of salivary gland stock of MCMV ($10^{7.3}$ p.f.u./ml) in DMEM for 1 hr adsorption at 37^{0}C in 5% CO_2. The cells were then used as target cells in a 6 hrs cytotoxicity assay with BALB/c MCMV-immune T cells. Control BALB/c MEF target cells were either untreated or infected with a 1/8 dilution of salivary gland stock MCMV in DMEM using the centrifugation protocol and incubated for 16 hrs prior to addition of MCMV-immune T cells.

Control MCMV-infected BALB/c MEF were susceptible to lysis by syngeneic MCMV-immune T cells (Table 5) whereas MCMV-treated PEC were not.

Susceptibility of BALB/c PEC treated with tissue culture stock of MCMV to lysis by syngeneic MCMV-immune T cells

$10^{5.7}$ BALB/c PEC in 125 ul assay medium were added to 1 ml of neat, 1/3 or 1/9 dilution of tissue culture stock MCMV ($10^{6.3}$ p.f.u./ml) in DMEM for 1 hr adsorption at 37^{0}C in 5% CO_2. The subsequent steps were identical to the protocol described for salivary gland stock MCMV adsorption to PEC. Control BALB/c MEF were either untreated or infected with a 1/8 dilution of salivary gland stock of MCMV ($10^{7.3}$ p.f.u./ml) in DMEM using the centrifugation protocol and incubated for 16 hrs prior to addition of

MCMV-immune T cells. Six hours after mixing of target and effector cells, cell-free supernatants were harvested and assayed for gamma emissions.

PEC treated with tissue culture stock of MCMV were lysed by MCMV-immune T cells (Table 6). The amount of cytotoxicity was improved by increased viral input.

DISCUSSION

Initial experiments compared susceptibility to lysis by MCMV-immune T cells of MEF treated with salivary gland stock of MCMV and similarly treated BALB/c-3T3, P815, PU5-1.8. MEF were superior to the other cells tested for the detection of lysis of MCMV-treated target cells above the lysis obtained on untreated cells. Consequently, MEF and salivary gland MCMV were the starting point for the further manipulation of conditions that resulted in changes in susceptibility of infected MEF to T cell-mediated lysis.

Since antiviral T cell function is dependent upon the recognition of H-2 and viral antigens (Zinkernagel & Doherty 1979), procedures calculated to improve the expression of these antigens on target or stimulator cells were investigated. Culture of various cell lines in medium supplemented with CSS increases H-2 antigen levels, as measured by increased surface binding of fluorescein labelled anti-H-2 antibodies (Wong et al. 1983). In our hands culture of MEF in CSS supplemented DMEM similarly increased binding of fluorescein labelled antibody specific for Class I H-2 antigens (King et al., in press).

That this phenomenon also reached physiological significance for target T cell interactions was suggested by the increased susceptibility of CSS-treated MEF compared with untreated MEF to lysis by alloreactive T cells (Table 2). Since at a fixed effector:target ratio more ^{51}Cr was released from CSS-treated than from untreated MEF it appeared that CSS treatment of MEF caused more alloreactive T cell-MEF interactions to result in lytic events.

This increased susceptibility to lysis was maintained for at least 16 hrs after removal of CSS-supplemented medium (data not shown). Although it is not possible to exclude that the enhanced levels of apparent lysis are merely an indication of a physiological alteration in ^{51}Cr release, the increased ability of CSS-treated MEF to stimulate IL-3 release from T cells (Fig. 1) makes this explanation unlikely.

For a fixed number of alloreactive T cells, CSS-treated MEF stimulated more IL-3 release than untreated MEF. In addition, more IL-3 was released for each unit increase in the number of CSS-treated MEF stimulators compared with untreated MEF (Fig. 1). These observations suggest that more alloreactive T cell-stimulator cell interactions resulted in IL-3 release or that more IL-3 per T cell was released by stimulation with CSS-treated MEF than untreated controls.

Since genetically determined differences in H-2 antigen expression of a magnitude similar to those produced by CSS treatment lead to differences in T cell-mediated lysis of target macrophages, or stimulator ability of splenocytes (O'Neill & Blanden 1979) it seems likely that increased H-2 antigens expressed on CSS-treated MEF (King et al., in press) led to their improved recognition by T cells.

The total number of MEF susceptible to lysis by MCMV-immune T cells and the amount of ^{51}Cr released at a given effector:target ratio were increased if MEF were adsorbed with MCMV under centrifugation (Table 3). This may be explained by an increase in the number of virions entering each MEF under centrifugation (Osborn & Walker 1968) but other unknown factors may be involved.

Viral titrations undertaken using the centrifugation protocol showed increased MCMV-immune T cell-mediated lysis of infected MEF targets with increasing virus input into the target cells (Fig. 2) particularly if CSS-treated MEF were used. This would suggest that susceptibility to lysis of CSS-treated MEF is limited by virus input-related parameter(s) whereas susceptibility of CSS-untreated, MCMV-infected MEF may be more limited by cellular factors sensitive to enhancement by alteration of culture conditions. One such candidate factor may be the level of H-2 antigen expressed at the cell surface.

With regard to the time taken after MCMV adsorption for MEF to acquire susceptibility to lysis by MCMV-immune T cells, susceptibility was detectable at 2 hrs post infection, was higher at 4 hrs and did not significantly increase by 18 hours. The ability to detect susceptibility to lysis by 2 hrs post-adsorption is similar to the findings for ectromelia virus (Ada et al. 1976) and for HSV (Pfizenmaier et al. 1977) where susceptibility to lysis occurred within 60 and 90 mins post adsorption respectively.

MEF appear to express only Class I H-2 and not Class II, H-2 antigens on their cell surfaces (King et al., unpublished observations); thus they are inadequate for the detection of Class II H-2 restricted T cells. However, macrophage populations contain Class II antigen-positive cells (Steeg et al. 1982) and have been successful targets for T cell recognition using other viruses (Zinkernagel & Doherty 1975). Furthermore, peritoneal macrophages may be capable of MCMV infection as assessed by p.f.u. or infectious centre assays (Tegtmeyer & Craighead 1968).

Further experiments are required to establish if these cells will act as MCMV-specific targets or stimulator cells for Class II

restricted T cells.

In vitro assessment of peritoneal macrophage infection by salivary gland or tissue culture passaged MCMV stock using fluorescent antibody was conducted by Mims & Gould (1978) and indicated that tissue culture passaged MCMV had more ability to infect peritoneal macrophages than salivary stock. Our results using MCMV-immune T cells (Tables 5 & 6) are consistent with the findings of Mims & Gould (1978). PEC treated with tissue culture stock MCMV were susceptible to lysis by MCMV-immune T cells (Table 6). The maximum levels of cytotoxicity and the susceptibility to lysis at a fixed effector aliquot were directly related to the viral input of tissue culture virus stock used during adsorption. PEC treated with salivary gland MCMV stock at all dilutions were not susceptible to lysis. The total inability of salivary gland MCMV stock to confer susceptibility to lysis upon broth-stimulated PEC in our hands may be explained by the higher resistance to MCMV infection of broth-stimulated peritoneal macrophages compared to resident peritoneal macrophages (Mims & Gould 1978). That susceptibility of PEC to lysis by MCMV-immune T cells was obtained within 7 hrs of tissue culture stock MCMV adsorption (Table 6), compared with 24-48 hrs after adsorption required for MCMV-specific antiserum to detect viral antigen in cells (Mims & Gould 1978), suggests either that MCMV-immune T cells may be more sensitive indicators of MCMV-specific antigens or may be capable of detecting MCMV-specific antigens not detected by hyperimmune anti-MCMV mouse serum, as also suggested previously in the case of alpha virus infected cells (Müllbacher et al. 1979).

SUMMARY

The preparation of target cells susceptible to lysis by MCMV-immune T cells *in vitro* was investigated and found to be dependent upon target cell type, culture conditions, virus adsorption protocol and virus preparation. Optimally, sensitive MCMV-infected targets were obtained by preculture of MEF in 3% vol/vol CSS-supplemented medium, adsorption of salivary gland stock MCMV under 800g centrifugation and at least 4 hrs further incubation at 37^{0}C before addition of T cells. In contrast, salivary gland stock MCMV did not cause thioglycollate-induced peritoneal exudate cells to be susceptible to MCMV-specific T cell-mediated lysis, whereas tissue culture-passaged stock MCMV was successful. The preparation of MCMV-infected target cells is discussed in terms of the need for H-2 and viral antigen expression for T cell recognition.

TABLE 1

Susceptibility of different H-2^d MCMV-treated target cells to lysis by BALB/c (H-2^d) MCMV-immune T cells*

	Target cell type	% lysis of target cells†	
		MCMV-treated‡	Untreated
Expt 1	BALB/c MEF	29.1 ± 0.4	5.9 ± 0.5
	P815	21.3 ± 1.1	24.5 ± 0.7
Expt 2	BALB/c MEF	52.6 ± 3.3	18.5 ± 0.6
	BALB/c-3T3	16.5 ± 3.3	7.8 ± 1.1
	PU5-1.8	54.1 ± 8.6	40.0 ± 3.1

* MCMV-immune T cells were generated from popliteal LN of BALB/c female mice inoculated into both hind f.p. with $10^{4.6}$ p.f.u. MCMV. Day 7 popliteal LN cells were removed and cultured for 4 days.

† % lysis represents the mean ± standard error of the mean of triplicate assays obtained by addition of a 1/12 aliquot of cultured cells from 6 BALB/c popliteal LN in a 10 hrs assay.

‡ Target cells exposed to virus as described in Methods.

TABLE 2

Effect of culture in CSS-supplemented medium upon susceptibility of BALB/c MEF to lysis by alloreactive T cells*

Effector:target ratio	% lysis of BALB/c MEF†	
	‡CSS+	CSS-
30:1	78.6 ± 1.6	58.7 ± 1.4
10:1	76.2 ± 2.3	53.1 ± 1.7
3.3:1	60.1 ± 3.4	19.5 ± 1.4
1.1:1	32.3 ± 2.3	8.1 ± 0.5

* Alloreactive T cells were generated from mixed lymphocyte cultures of C57Bl/6J mesenteric LN cells and irradiated BALB/c spleen cells according to Woolnough & Lafferty (1979).

† % lysis represents the mean ± standard errors of the mean of triplicate assays obtained by addition of effectors.

‡ BALB/c MEF were cultured in medium supplemented with or without 3% vol/vol CSS for 48 hrs prior to use as targets.

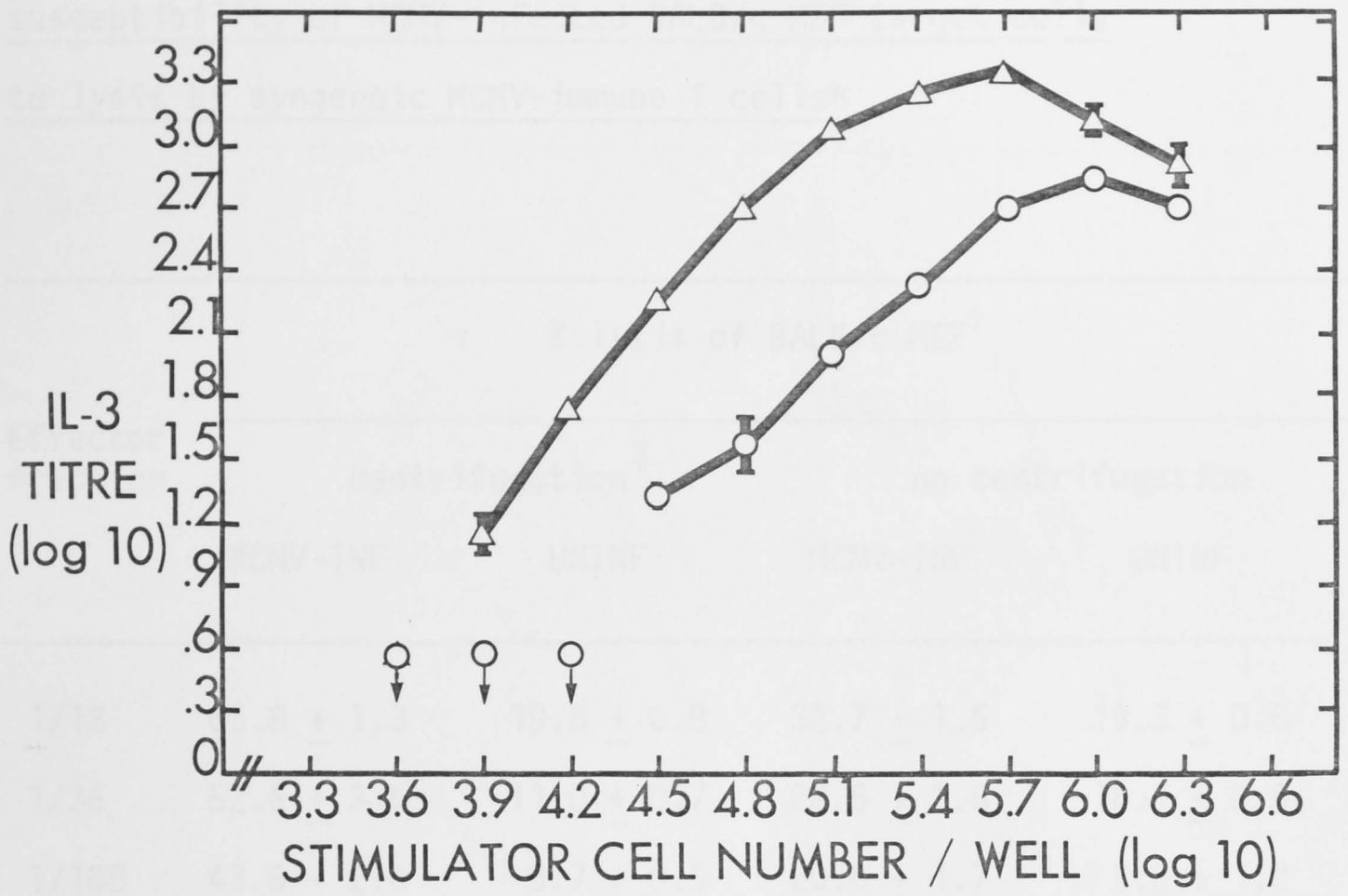

Fig. 1. Curve showing the effect of culture in CSS-supplemented medium upon the ability of BALB/c MEF to stimulate IL-3 release from alloreactive T cells. BALB/c MEF were cultured for 48 hrs in medium supplemented with (△) or without (○) 3% vol/vol CSS. $10^{5.7}$ alloreactive T cells, generated as described in Materials and Methods, were mixed with different numbers of BALB/c MEF stimulator cells for 6 hrs, after which time cell-free supernatants were harvested and assayed for IL-3. Each point represents the mean and standard deviation of the mean IL-3 titre of quadruplicate estimations. Where no bars are indicated the standard deviations are within the points. (○↓) represent IL-3 levels below detection limits.

TABLE 3

Effect of centrifugation during viral adsorption upon susceptibility of MCMV-infected BALB/c MEF target cells to lysis by syngeneic MCMV-immune T cells*

Effector fraction	% lysis of BALB/c MEF†			
	centrifugation‡		no centrifugation	
	MCMV-INF	UNINF	MCMV-INF	UNINF
1/12	65.8 ± 1.3	19.8 ± 0.8	32.7 ± 1.5	19.3 ± 0.6
1/36	52.6 ± 3.5	11.0 ± 0.7	28.6 ± 2.6	16.1 ± 0.9
1/108	43.6 ± 2.0	5.7 ± 0.5	20.0 ± 1.7	8.8 ± 1.2
1/328	26.3 ± 1.6	2.6 ± 0.1	13.2 ± 1.0	5.6 ± 0.5

* As for Table 1 except mice were inoculated with $10^{2.6}$ p.f.u. MCMV into hind f.p.

† As for Table 1 except syngeneic MEF were cultured in medium supplemented with 3% vol/vol CSS prior to MCMV infection and addition of aliquots of MCMV-immune T cells.

‡ As for Table 1 except for centrifugation as described in Results.

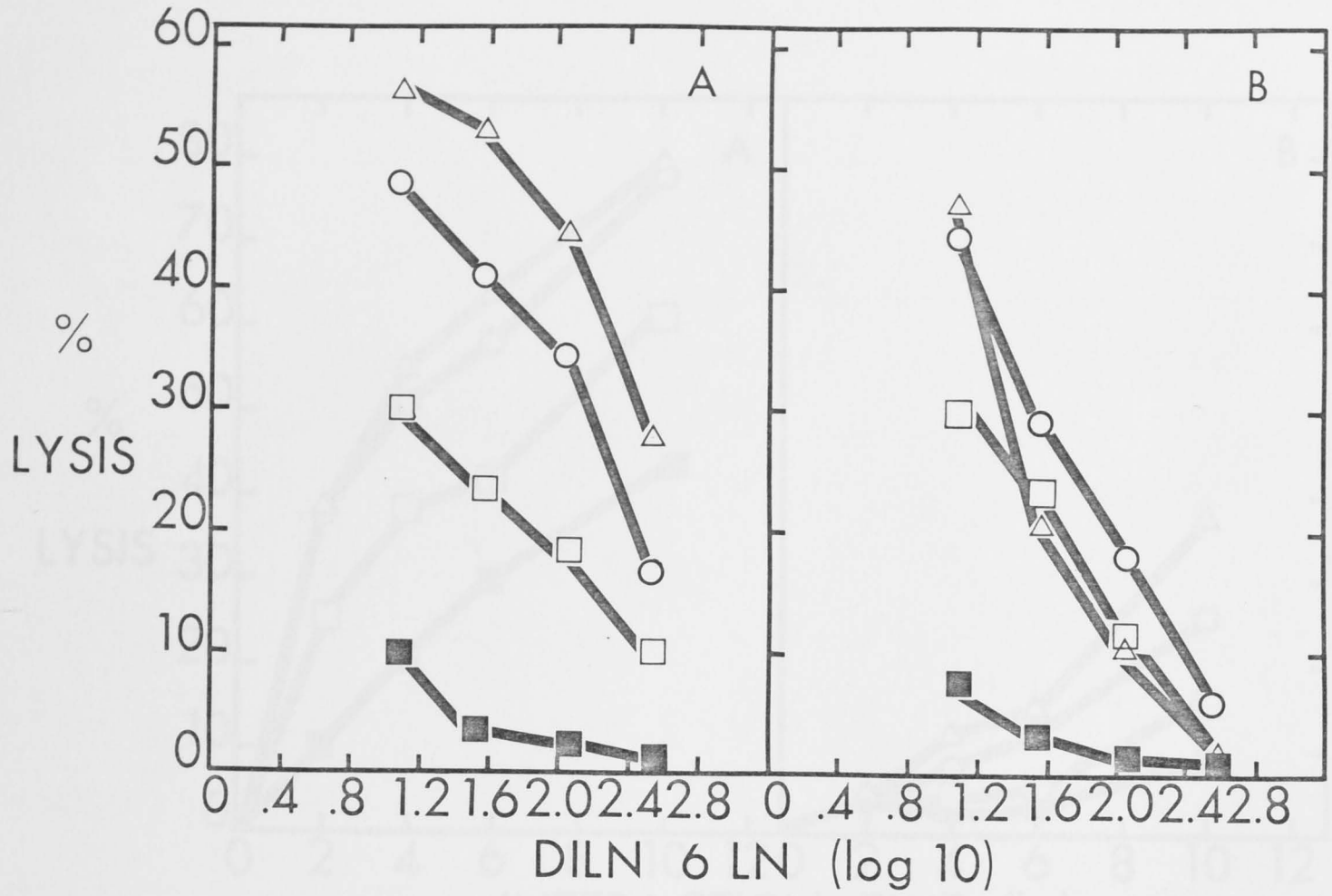

Fig. 2. Curves showing the effect of culture in medium supplemented with CSS upon susceptibility of BALB/c MEF to lysis by syngeneic MCMV-immune T cells. BALB/c MEF were cultured for 48 hrs in medium with (A) or without (B) added CSS and infected with salivary gland stock MCMV, diluted 1/8 (△), 1/16 (○) and 1/32 (□) in DMEM, or left uninfected (■). % lysis represents the mean of triplicate assays obtained by the addition of MCMV-immune T cells to MEF target cells for 10 hrs. The standard errors of the mean were <4% and are not shown.

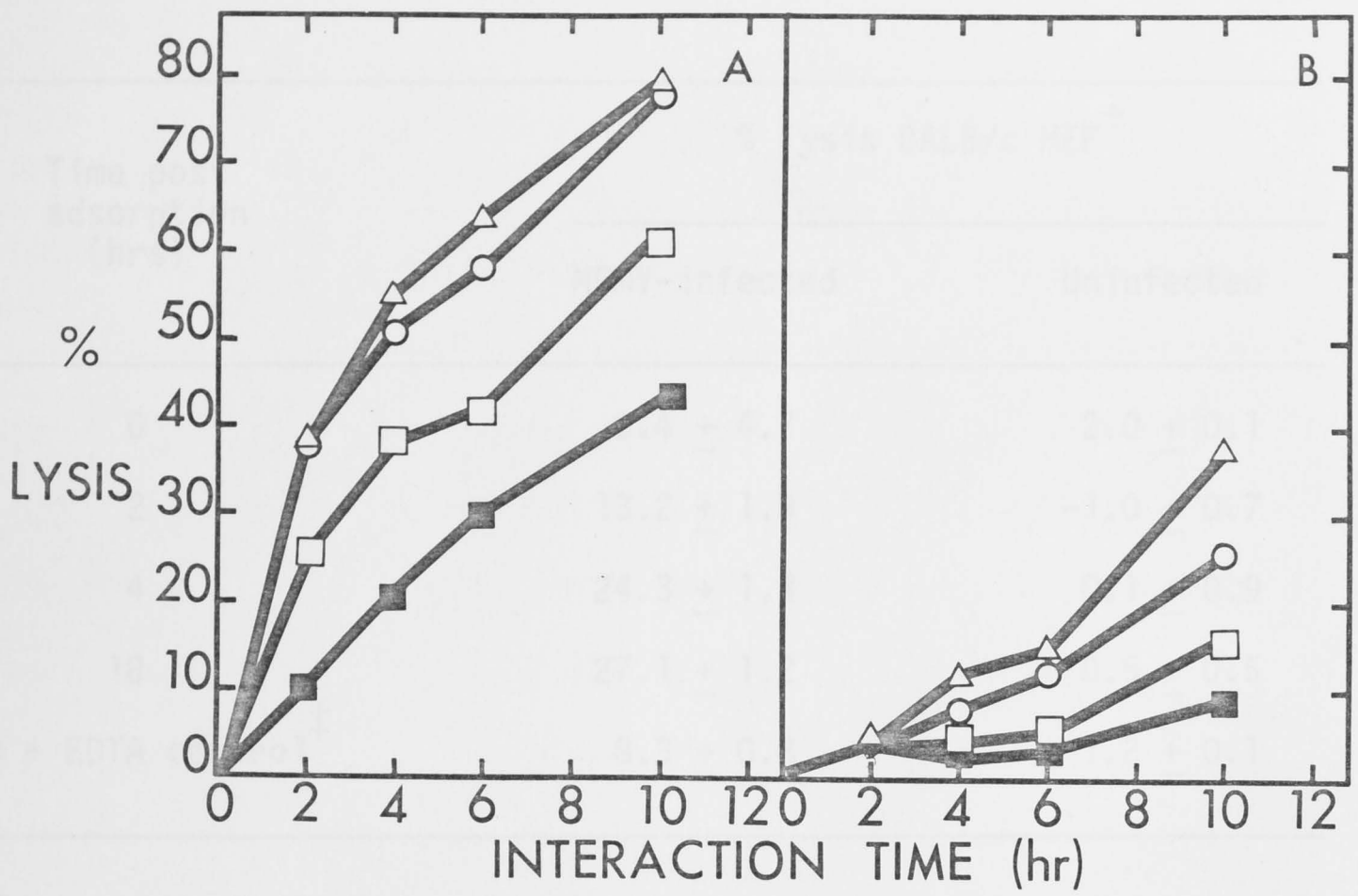

Fig. 3. Curves indicating the kinetics of ^{51}Cr release from MCMV-infected or uninfected BALB/c MEF following addition of different numbers of MCMV-immune T cells. BALB/c MEF were infected with 1:8 dilution of salivary gland stock MCMV in DMEM for 16 hrs (A) or left uninfected (B). 1/12 (△), 1/36 (○), 1/108 (□) and 1/324 (■) dilutions of MCMV-immune T cells were added and supernatants removed at the times indicated. % lysis represents the mean of triplicate assays. The standard errors of the mean were $<4\%$ and are not shown.

TABLE 4

Effect of time after MCMV adsorption upon the susceptibility of MCMV-infected BALB/c MEF to lysis by syngeneic MCMV-immune T cells *

Time post adsorption (hrs)	% lysis BALB/c MEF†	
	MCMV-infected	Uninfected
0	0.4 ± 4.7	2.0 ± 0.1
2	13.2 ± 1.9	-1.0 ± 0.7
4	24.3 ± 1.3	0.1 ± 0.9
18	27.1 ± 1.2	0.5 ± 0.5
18 + EDTA control‡	8.3 ± 0.8	-1.2 ± 0.1

* As for Table 3.

† % lysis represents the mean ± standard error of the mean of triplicate assays obtained by the addition of a 1/36 aliquot of cultured cells from 6 BALB/c popliteal LN. Forty-five mins after mixing of targets and MCMV-immune T cells 0.04M EDTA was added. Cell free supernatants were harvested after a further 4 hrs.

‡ As for † except EDTA containing medium was added immediately after MCMV-immune T cells and syngeneic MEF were mixed.

TABLE 5

Susceptibility of BALB/c MEF and PEC infected with salivary gland stock MCMV to lysis by syngeneic MCMV-immune T cells*

Cell type and MCMV stock dilutions	% lysis†	
	MCMV-treated cells‡	Untreated cells
MEF 1:8	39.8 ± 0.6	3.6 ± 0.2
PEC 1:4	0.8 ± 4.4	-11.0 ± 1.7
PEC 1:8	8.6 ± 12.7	-11.0 ± 1.7
PEC 1:16	3.3 ± 0.7	-11.0 ± 1.7
PEC 1:32	-10.2 ± 2.5	-11.0 ± 1.7

* As for Table 3.

† As for Table 1 except that a 1/36 aliquot of MCMV-immune T cells was added in a 6 hrs assay.

‡ BALB/c MEF and PEC were treated with dilutions of salivary gland stock MCMV as described in Methods and Results.

TABLE 6

Susceptibility of BALB/c MEF PEC treated with tissue culture stock of MCMV to lysis by MCMV-immune T cells*

Cell type and MCMV stock dilutions		% lysis†	
		MCMV-treated cells‡	Untreated cells
MEF	1:8	34.9 ± 0.2	6.1 ± 0.5
PEC	NEAT	55.0 ± 2.7	4.1 ± 0.2
PEC	1:3	44.4 ± 1.2	4.1 ± 0.2
PEC	1:9	35.0 ± 0.9	4.1 ± 0.2

* As for Table 3.

† As for Table 5.

‡ BALB/c MEF and PEC were treated with dilutions of tissue culture or salivary gland stock of MCMV as described in Methods and Results.

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CHAPTER 6

DISCUSSION AND SUMMARY

The work presented in this thesis was undertaken to provide more information regarding T lymphocyte responses to murine cytomegalovirus (MCMV) infection.

That T cells can trigger the elimination of MCMV infection was established by the adoptive transfer experiments of Starr & Allison (1977) and Ho (1980). The results of these workers do not differ from adoptive transfer data obtained using other acute viral infection models (Zinkernagel & Doherty 1979). However, where infection with MCMV does differ from these other models is in the occurrence of latent infection and reactivation. Two hypotheses can be entertained to explain the latency phenomenon. (i) The induction of anti-MCMV T lymphocyte responses and antibodies or the effector mechanisms mediated by such responses are inadequate for complete viral elimination. (ii) The ability of virus to remain within a mouse possessing a normal immune system is a peculiarity of virus-host interaction determined by non-immunological factors.

An obvious instance of the latter would be infection of a known immunologically privileged site (Billingham & Boswell 1953; Bacsich & Wyburn 1956; Burnet 1959) with resultant failure of cell activation and viral elimination. With regard to cell activation this certainly does not apply to MCMV since many non-privileged organs are infected (Hudson 1979) and an immune response, albeit weak, is generated rather than completely absent (Quinnan et al. 1978; Osborn et al. 1968; Mannini & Medearis 1961). However, the privileged site argument may be used to explain the ability of MCMV to remain within spleen cells, as judged by DNA-DNA hybridization or allogeneic co-cultivation techniques in the presence of an activated immune system (Olding et al. 1976; Brautigam et al. 1979).

Since the normal function of the virus-specific T cell is dependent upon recognition of both cell surface viral and H-2 antigens the privileged sites could also arise if H-2 antigen-negative cells were MCMV-infected; viral infection modulated the level of H-2 antigens expressed at the cell surface, or infected cells displayed no cell surface viral antigens.

In this regard it would be of interest to include cell surface H-2 antigen levels in the phenotypic characterisation of cells known to harbour MCMV genome.

Dutko & Oldstone (1981) showed certain undifferentiated cells did not display viral antigens when infected with MCMV, until chemically induced to undergo differentiation. In this instance, although cell differentiation was assessed by the cell surface levels of H-2 antigen, a cause and effect relationship between viral antigen and H-2 antigen expression was not implied.

On the other hand, can MCMV-infection of cells result in decreased H-2 antigen levels at the cell surface? Preliminary investigations showed decreased binding of anti-H-2^d antiserum and decreased susceptibility to lysis by anti-H-2^d effector T cells of MCMV-infected BALB/c mouse embryo fibroblasts (MEF) compared to uninfected syngeneic MEF (unpublished observations, King & Sinickas). These results suggested that H-2 antigen levels on MEF surface membranes were reduced during MCMV infection. Similar observations have been reported for ectromelia-infected target cells (Gardner et al. 1975).

Environmental factors may also affect the level of cell surface H-2 antigens. In Chapter 4 it was demonstrated that culture of BALB/c MEF in medium supplemented with Concanavalin-A

stimulated spleen cell supernatants (CSS) produced increased susceptibility to lysis and increased stimulator ability by alloreactive T cells, compared with control syngeneic MEF. Similarly, *in vitro* susceptibility to lysis of virus-infected MEF target cells, by MCMV-immune syngeneic T cells, was increased following growth of MEF in CSS-supplemented medium prior to viral adsorption. Hence T cell recognition of virus-infected target cells was apparently not limited by viral input but rather by some factor(s) related to MEF culture conditions. It seems reasonable to suggest that the known increase in H-2 antigen levels of MEF in CSS-supplemented medium is responsible for the increased susceptibility to lysis. Although it is theoretically possible that increased viral receptors and therefore, increased viral input per target cell, may account for the generation of increased susceptibility to lysis, there is no precedent for this hypothesis. An experimental test of the latter hypothesis would show CSS-treated MEF generated increased viral plaque forming units (p.f.u.) after MCMV adsorption compared with CSS-untreated MEF.

During acute primary infection with MCMV or Herpes simplex virus (HSV) the development and activity of virus-specific T cells can be assessed by a variety of detection systems that include adoptive transfer of protection from or elimination of virus infections (Starr & Allison 1977; Ho 1980), delayed-type hypersensitivity reactions (Chong & Mims 1982), T cell-dependent antibody formation (Oakes 1975; Burns *et al*. 1975), *in vitro* cytotoxicity (Quinnan *et al*. 1978; Pfizenmaier *et al*. 1977a) and lymphokine production (Chapter 4). However, the inability to generate easily detectable virus-specific cytotoxic T cell responses (over and above cytotoxicity on uninfected targets) during acute primary

infection with MCMV or HSV, has provided a major obstacle to investigation of the T cell response. This aspect is additionally puzzling since the adoptive transfer experiments clearly showed MCMV elimination *in vivo* was Class I, H-2 restricted, suggesting the participation of cytotoxic T cells generated in the donor mice during immunization with MCMV (Ho 1980). With HSV infection T lymphocytes are also important, as discussed previously, but which T lymphocyte subsets are involved in recovery is a little less clear. Adoptive transfers (intravenously) of *in vitro*-restimulated HSV-primed spleen cells into recipients inoculated intraperitoneally, with a lethal dose of HSV 24 hours previously, produced decreased mortality rates compared with HSV-inoculated recipients of cultured normal spleen cells (Larsen *et al*. 1983). Since the cultured HSV-primed spleen cells contained cytotoxic T cells specific for HSV the correlation of cytotoxicity and reduced mortality indicated a possible role for such cells.

However, intravenous transfer of donor lymph node cells with no detectable cytotoxic T cell activity, obtained from mice inoculated with HSV into the ear 7 days previously, resulted in lower auricular HSV titres in recipient mice compared with animals that received no cells (Nash *et al*. 1980a,b). The antiviral effect was mediated by $Lyt1^{+}2^{-}$ cells (Nash & Gell 1983). With the protocol utilised (transfer of immune cells into recipients one hour prior to HSV inoculation) the possibility exists for HSV-immune cells to interact with the viral inoculation and alter the events at the inoculation site that lead to productive infection and replication. To exclude this, the temporal sequence of viral inoculation and cell transfer should be reversed and daily monitoring of viral p.f.u. in the ear obtained to

show the onset of viral elimination. The abovementioned possibility was obviated to some degree by Larsen et al. (1984) who confirmed that similar cell phenotypes were responsible for HSV clearance from ears of cyclophosphamide treated recipients of intravenously transferred HSV-immune cells. The immune cells were obtained from donor mice 7 days after subcutaneous inoculation of HSV and transferred into recipients 2 hours after HSV inoculation into the ear. However, the immune responses to HSV in cyclophosphamide-treated mice may be altered (Pfizenmaier et al. 1977b; Rager-Zisman & Allison 1976) and mitigate interpretation of cell phenotypes responsible for HSV elimination. The interpretations may be further compounded by the time intervals (3 to 4 days) involved before assessment of the effect of cell transfer upon viral elimination. Experimentation with cyclophosphamide untreated mice, inoculated with HSV prior to adoptive cell transfers and assayed for viral p.f.u. 24 hours after infection must be performed. In addition, since anti-Lyt1$^+$ antiserum and complement (C') treatment has the potential to eliminate Lyt1$^+$2$^+$ cells it is not possible to exclude a role for Lyt2$^+$ cells in the generation of lower viral p.f.u. until such cells are positively selected, transferred and effects assessed.

Two broad hypotheses could account for the inability to detect HSV or MCMV virus-specific cytotoxicity. Either (i) the virus-specific cytotoxic response was quantitatively lower for MCMV than for other non-Herpesviridae or (ii) the detection system for MCMV-specific cytotoxicity was a less sensitive assay than the viral elimination techniques used to detect the presence of Class I, H-2 restricted T cells. With HSV, it is also possible that the targets

utilised to detect cytotoxicity are inappropriate. Swain (1983) suggested Lyt phenotype correlated with MHC antigen class recognised by T cells rather than T cell function. $Lyt2^+$ T cells recognised Class I MHC antigens on cell surfaces whilst $Lyt2^-$ T cells recognised Class II MHC antigens. Therefore if HSV-immune T cells generated during HSV infection are of $Lyt2^-$ phenotype (Nash & Gell 1983, Larsen et al. 1984), and are cytotoxic, the targets utilised for detection of cytotoxicity must possess viral antigens and Class II MHC molecules. BALB/c MEF do not possess Class II MHC as judged by the inability to bind fluorescent labelled anti-IA^d antiserum (King & Sinickas, unpublished observations). Hence target cells such as MEF, and other Class II^- cells may be inappropriate for detection of $Lyt2^-$ antiviral T cells. That Class II restricted cytotoxicity can occur was demonstrated with human cytotoxic T cell clones against HSV (Yasukawa & Zarling 1984). However, since many cells in the body are Class II^- the usefulness of such T cells as antiviral effectors would be limited.

As a first step to examining the T cell response to MCMV a reproducible technique for MCMV-immune T cells generation and cytotoxicity testing was sought. The model of hind footpad (f.p.) inoculation with virus, removal and culture of draining popliteal lymph node (LN) cells described for the generation of HSV-specific cytotoxic T cells (Pfizenmaier et al. 1977a) was adapted to MCMV. The data in Chapters 2 and 3 confirmed the usefulness of this model for the generation of MCMV-specific cytotoxicity dependent upon $Thy1^+$, $Lyt2^+$, Class I, H-2 restricted cells, the difficulty in detection of MCMV-specific cytotoxicity directly from virus-stimulated lymphoid cell populations was confirmed (Ho, 1980) and

was at variance with Quinnan et al. (1978, 1980). Even when putative cold-target competitor cells, (Immunoglobulin$^+$ cells), were removed in an attempt to enhance detection levels, no cytotoxicity was observed.

For detection of MCMV-immune T cells, MCMV inoculation into the f.p. followed by a minimum of 4 days in vivo prior to removal of draining popliteal LN cells and 2 days of culture was required, but optimal MCMV-specific cytotoxicity was generated by removal of draining popliteal LN cells 6 to 8 days after MCMV f.p. inoculation and 4 days in vitro. Cellular proliferation in vitro was essential for generation of cytotoxicity and was dependent upon a Thy1.2$^+$ cell population. This suggests that the levels of cytotoxicity are not regulated by a lack of precursors with potential for cytotoxicity but rather a lack of expression of this potential.

During the course of the investigation Reddehase et al. (1984), using immune cells derived by a similar protocol, reported the precursor frequencies of cells cytotoxic to MCMV-infected MEF and showed two cell populations were present in vitro. One population developed cytotoxic activity without added MCMV antigen whilst the other, more frequent, cell population was dependent upon added MCMV-infected syngeneic MEF during culture. Since not all the cytotoxic clones generated in vitro were split and assayed on MCMV-infected and uninfected syngeneic MEF some of the clones may have been autoreactive rather than virus specific. Autoreactivity, i.e. cytotoxicity of uninfected self targets, was noted in Chapter 2 with maximum levels occurring between day 2 to day 6 of culture when lymph node cells were removed 4 days after f.p. inoculation of MCMV. With this reservation in mind the total precursor

frequency of T cells reactive to MCMV antigens in day 8 popliteal lymph nodes was 1:2700-1:7200, figures of the same order of magnitude as precursor frequencies noted during acute infections with rabies virus (Reddehase et al. 1982) and similar to frequencies seen in primed spleen populations restimulated in vitro with minor histocompatibility antigens (Teh et al. 1982), male alloantigens (Kanagawa et al. 1982), murine sarcoma virus-induced tumours (Brunner et al. 1980) and influenza (Askonas et al. 1982). This again suggests that lack of direct cytotoxicity is not due to a lack of or low levels of precursor T cells against MCMV-infected cells.

Hence, either the clonal expansion of MCMV-immune T cells or the maturation to the cytotoxicity function of T cells or both, were required prior to detection in vitro. A need for clonal expansion of MCMV-immune T cells to detectable levels is favoured because (i) optimal cytotoxicity was dependent upon proliferation of $Lyt2^+$ cells and (ii) the addition of CSS during culture enhanced the levels of cytotoxicity attained.

Two major events are involved in the generation of a T cell response. Induction, mediated by both antigen recognition and a second Interleukin-1 (IL-1)-mediated inductive signal, and T cell proliferation dependent upon Interleukin-2 (IL-2). To determine whether soluble factor(s) in CSS were required for maturational events in vitro leading to cytotoxicity, a purified source of IL-2 would be required, thus allowing T cell proliferation to take place in the absence of these putative factors.

Two models are available to explain activation and clonal expansion of antigen or mitogen-reactive T cells (Larsson et al. 1980;

Lafferty et al. 1980). Both models propose antigen recognition and IL-1-mediated signals are necessary for induction of a T cell response but differ in the origin of the IL-2 necessary for clonal expansion of effector T cell populations. Larsson et al. (1980), Wagner et al. (1980) suggest that a special class of T cells (T helpers) respond to antigen and IL-1 and then provide the IL-2 necessary for activation and clonal expansion of a separate class of responding cells, the precursors of cytotoxic T cells. Hence, the model proposes the existence of two functionally distinct T cell subsets with different activation requirements; a helper subset which is IL-1 dependent, and an IL-2 dependent effector subset, incapable of IL-2 synthesis. The T helper subset has been equated phenotypically with the $Lyt2^-$ T cell subset (Wagner et al. 1980).

On the other hand, Lafferty et al. (1980) do not agree with the division of responder T cell populations into separate helper and effector subsets but rather postulate all T cells are capable of both producing and responding to IL-2 and of expressing other effector functions such as cytotoxic activity.

If the former model is correct as would be suggested by the requirement of $Lyt2^-$ cells for the in vitro generation of anti-influenza (Müllbacher & Ashman 1979) or anti-ectromelia cytotoxic T cells (Pang et al. 1976), the data in Chapter 3 indicating that there are low numbers of activated virus-specific $Lyt2^-$ cells in LN cell populations draining MCMV inoculation sites could account for low cytotoxic T cell responses. MCMV-immune LN cell populations depleted of $Lyt2^+$ cells were delayed in thymidine incorporation and did not achieve the same maximum levels as untreated or

C' treated control MCMV-immune LN cells. Inadequate non-antigenic culture conditions, or viral inhibition of responses were excluded by the equivalent proliferative responses to Concanavalin-A of $Lyt2^+$-depleted and untreated MCMV-immune LN cells.

Table 1 in Chapter 4 argues against complete absence of $Lyt2^-$ MCMV-specific T cells since Interleukin-3 (IL-3) release from MCMV-immune T cells incubated alone, was not abrogated by anti-Lyt2 antiserum and C' treatment as was the case with anti-Thy1.2 antiserum and C' treatment. Furthermore data from HSV infections indicates the presence of $Lyt2^-$ cells since auricular LN cells or spleen cells removed from mice on day 6 after subcutaneous inoculation of virus and treated with anti-Lyt2 antiserum and C' can successfully transfer HSV-specific delayed-type hypersensitivity to unprimed recipients (Nash & Gell 1983; Larsen et al. 1984). With MCMV, although adoptive transfer and phenotypic characterisation have not yet been performed, the ability to successfully generate and elicit delayed-type hypersensitivity responses with live and heat-inactivated MCMV preparations (Chong & Mims 1982) suggests the presence of $Lyt2^-$ cells. In addition, the antibody response to HSV is T cell dependent (Burns et al. 1975; Oakes 1975) and if its requirements are similar to antibody generation to other antigens then it would necessitate the presence of Class II H-2 restricted, $Lyt1^+2^-$ antigen specific T cells (Katz et al. 1973b; Sprent 1978a,b; Swierkosz et al. 1978).

The collective reports would then argue against complete absence of $Lyt2^-$ MCMV-specific precursor T cells but the data cannot address any quantitative variation in total numbers of $Lyt2^-$ cells or the amount of IL-2 released after stimulation or the

proliferative responses to IL-1 or antigen. These facets of MCMV-specific T cell responses will be amenable to investigation with target and stimulator cells, such as macrophages, that possess both Class II MHC and MCMV-specific antigens on the cell surface (Chapter 5).

In Chapter 4, MCMV-immune T cells were utilised to determine whether lymphokine production and cytotoxicity were mediated by the same or two separate T cell subsets. It was shown that IL-3 release was dependent upon MCMV-specific, Thy1.2^{+}, Lyt2^{+}, Class I, H-2 restricted cells which were phenotypically similar to MCMV-immune T cells required for virus-specific cytotoxicity. This data was consistent with the idea that the same virus-specific T cells performed both lymphokine release and cytotoxic functions, but anti-MCMV T cell clones would be required to unequivocally establish the fact. Hence the Lafferty et al. (1980) hypothesis would require that the low anti-MCMV cytotoxicity levels reside in either the inability of anti-MCMV T cells to release a particular lymphokine, IL-2, or in the inability of anti-MCMV T cells to proliferate in response to IL-2.

Although, T cell clones (Prystkowski et al. 1982) and hybridomas (Zlotnik et al. 1983; Kelso & Glasebrook 1984) are capable of multiple lymphokine production, including IL-3 and IL-2, subsequent to antigen or mitogen stimulation it is necessary to establish whether MCMV-immune T cells can indeed produce both IL-3 and IL-2. In fact it would be of interest to compare the spectrum of lymphokines released by MCMV-specific T cells with the spectrum produced by other antiviral T cells and to investigate variations in the spectrum of lymphokine production using different prepara-

tions of MCMV-infected cells as an indication of the effector potential of such T cells. Lin & Askonas (1981), Morris et al. (1982) and Taylor & Askonas (1983) established that both cytotoxic and lymphokine release functions, not just cytotoxicity alone, were important for in vivo elimination of influenza infection with anti-influenza Class I H-2 restricted T cell clones. Since no Class I H-2 restricted, non-cytotoxic, lymphokine-producing T cell clones were used the data did not indicate if lymphokine production alone was sufficient for viral elimination.

Finally, since T cell proliferation is also controlled by lymphokine receptor density (Robb et al. 1981; Cantrell & Smith 1984) and receptor density is determined by exposure to antigen, antigen levels must also modulate T cell proliferation (Cantrell & Smith 1983). For MCMV this would explain the additional detection of precursors of cytotoxic T cells subsequent to antigen exposure compared with the number detected without added antigen (Reddehase et al. 1984). In addition, the adoptive transfer experiments (Ho, 1980) with antigen-primed cells removed from donor mice and transferred into virus-inoculated animals can be explained by antigenic restimulation and subsequent clonal expansion of primed cells sufficient to mediate viral elimination. A prediction of this hypothesis would be the presence of detectable direct cytotoxicity from recipient tissues but mediated by T cells of donor origin.

It would also appear that heterogeneity exists with regard to the preparations of MCMV capable of stimulating a response. Ho (1980) reported MCMV-infected MEF irradiated with ultraviolet (UV) light stimulated more proliferation and cytotoxic T cell formation from MCMV-primed spleen cells than did γ-irradiated or

unirradiated MCMV-infected MEF. Furthermore, MEF infected with MCMV for 2 hours prior to UV-irradiation and subsequently used as stimulators did not generate virus-specific cytotoxic T cells, whereas MEF infected with MCMV for 18 hours prior to UV-irradiation and used as stimulators generated MCMV-specific cytotoxicity. On the other hand, Reddehase et al. (1984) used MCMV-infected MEF without UV- or γ-irradiation for stimulation of an acute MCMV-immune LN cell population to generate more virus-specific cytotoxicity. The data in Chapter 4 also indicate that MEF infected with MCMV for different times will stimulate different levels of lymphokine release from MCMV-immune T cells, whilst Chapter 5 shows target cell-type and virus preparation are important variables for the generation of virus-specific antigenic changes on cell surfaces which are recognised by T cells.

In conclusion it is postulated that the precursors of MCMV-specific T cells are present within the immune system and that the inductive requirements for these T cells are provided to some extent during acute MCMV infection but the signals required for clonal expansion are limited. Exactly why this limitation occurs is not yet resolved. It could be due to low levels of IL-2 release from activated MCMV-specific T cells, to low levels of IL-2 receptor density on T cells subsequent to antigen exposure, or to insufficient virus-specific antigen on stimulator cell surfaces to trigger both IL-2 release from and IL-2 receptor expression on T cells.

These particular possibilities can be addressed by the use of IL-2 release and anti-IL-2 receptor antibodies with MCMV-immune T cells stimulated by virus-specific antigenic changes on a variety of stimulator cells obtained from in vivo and in vitro sources.

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